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Alarming Immunity: A Wake up Call for Cd8 T Cells Using Genetic Adjuvants (il-33 and Isg15)

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Abstract

There exists a paramount need for effective vaccines against cancer, TB, malaria, HIV, HCV, and other chronic infections. The hope for long-term control of these important diseases ultimately may depend upon development of potent T cell-based therapeutic vaccines. However, current, licensed vaccines or capable vaccine platforms have not made a substantial impact on treatment of these conditions, likely due in part to poor CD8 T cell immune induction. Thus, identification of novel adjuvants to be deployed to induce effective T responses is an important area of research in T cell based vaccines. While multiple adjuvants have been readily identified which impact CD4 T cells, it has been a challenging task to identify adjuvants that can amplify CD8 T cell responses. In this regard, Interleukin 33 (IL-33) and Interferon Stimulated Gene 15 (ISG15) have emerged as immunomodulatory molecules facilitating the generation of TH1-mediated T cell immunity; however, their ability to function as vaccine adjuvants to enhance CD8 T cell immunity was not previously explored. Here we used a DNA-vaccination approach to investigate the effect of IL-33 and/or ISG15 on vaccine-induced CD8 T cell immune responses. My studies showed that both IL-33 and ISG15 served as effective vaccine adjuvants to enhance the antigen-specific, polyfunctional, and cytolytic effector CD8 T cell responses in vivo. Importantly, I demonstrate for the first time the efficacy of both IL-33 and ISG15 as DNA vaccine adjuvants in driving viral or tumor protective immunity. Consideration of our findings, combined with a further understanding of the functional roles of these molecular adjuvants in immune expansion, likely will aid in the development of therapies for augmenting T cell based responses against many infectious diseases and cancers.

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**ALARMING IMMUNITY: A WAKE UP CALL FOR CD8 T CELLS
USING GENETIC ADJUVANTS
(IL-33 AND ISG15)**

Daniel Villarreal

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**ALARMING IMMUNITY: A WAKE UP CALL FOR CD8 T CELLS USING
GENETIC ADJUVANTS**

(IL-33 AND ISG15)

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2015

Daniel Villarreal

FOR MY FAMILY,
WHO MADE DREAMS COME TRUE,
AND FOR MY PILLAR OF STRENGTH, REBEKAH JOY,
WHO IS MY PACIFIC AND CALIFORNIA GOLD! GOLD! GOLD!

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ABSTRACT

ALARMING IMMUNITY: A WAKE UP CALL FOR CD8 T CELLS USING GENETIC ADJUVANTS (IL-33 AND ISG15)

Daniel Villarreal

David B Weiner

There exists a paramount need for effective vaccines against cancer, TB, malaria, HIV, HCV, and other chronic infections. The hope for long-term control of these important diseases ultimately may depend upon development of potent T cell-based therapeutic vaccines. However, current, licensed vaccines or capable vaccine platforms have not made a substantial impact on treatment of these conditions, likely due in part to poor CD8 T cell immune induction. Thus, identification of novel adjuvants to be deployed to induce effective T responses is an important area of research in T cell based vaccines. While multiple adjuvants have been readily identified which impact CD4 T cells, it has been a challenging task to identify adjuvants that can amplify CD8 T cell responses. In this regard, Interleukin 33 (IL-33) and Interferon Stimulated Gene 15 (ISG15) have emerged as immunomodulatory molecules facilitating the generation of T_H1 -mediated T cell immunity; however, their ability to function as vaccine adjuvants to enhance CD8 T cell immunity was not previously explored. Here we used a DNA-vaccination approach to investigate the effect of IL-33 and/or ISG15 on vaccine-induced CD8 T cell immune responses. My studies showed that both IL-33 and ISG15 served as effective vaccine adjuvants to enhance the antigen-specific, polyfunctional, and cytolytic effector CD8 T cell responses *in vivo*. Importantly, I demonstrate for the first time the efficacy of both

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CHAPTER 1

GENERAL INTRODUCTION

*"If I have seen further it is by
Standing on the shoulders of giants."*

—Isaac Newton

Vaccination is one of the greatest triumphs of modern medicine. Immunization eradicated smallpox in 1976 and has nearly eradicated polio from this world (1,2). In addition, the substantial reduction of the number of incidences of measles, mumps, diphtheria, and tetanus worldwide are examples of the incontrovertible success of vaccines in the prevention and protection against infectious pathogens (2). Many of the aforementioned vaccines are based on live attenuated or whole-killed organisms, as they mimic a live infection without causing disease. These traditional vaccines occasionally have well-known safety issues such as reversion, incomplete inactivation of virus and/or transmission of virus to immunocompromised individuals that deem them inappropriate for certain populations. Thus, alternative vaccine formulations, such as subunit, conjugate, toxoid and DNA vaccines, which contain only target proteins, are used to reduce the drawbacks concerning traditional vaccines. However, these alternative vaccine approaches are often poorly immunogenic as a result of its limited antigenic targets. Therefore, the addition of adjuvants has become a key method for enhancing the protective immunity generated by current vaccination.

Adjuvants

Adjuvant is derived from the Latin word *adjuvare* which means to help. It was first termed by Ramon Gaston, a French veterinarian, who defined it as a substance that enhanced immune responses to an antigen (3). Subsequently, Gleeny and colleagues would be the first to report the critical role adjuvants played in the effectiveness of vaccines. Gleeny *et al.* demonstrated the adjuvant activities of aluminum compounds, observing a 1,000 fold increase in antibody production when using an alum precipitated diphtheria toxoid vaccine (4,5). Since then, aluminum based adjuvants (aluminum hydroxide, aluminum phosphate, aluminum sulfate (alum)) have been used successfully

in vaccines for over 80 years for promoting the enhancement of antibody responses. Today, alum adjuvants can be found in several vaccines, such as Gardasil (HPV), DTaP, HBV, Haemophilus influenza B (HIB) and pneumococcal vaccines (Table 1) (6). Adjuvants currently licensed to be used in human vaccines in the US and/or Europe include aluminum-based salts, oil-in-water emulsions (MF59, AS03) and AS04 (monophosphoryl lipid A preparation (MPL) with aluminum salt) (**Table 1.1**). Due to the attractive property of adjuvants to promote and modulate vaccine immunogenicity they have become an increasingly important ingredient in novel vaccines being developed today.

Adjuvants, in the context of vaccines, are defined as substances that enhance and/or shape antigen (Ag)-specific immune responses. For new vaccines in development, adjuvants are increasingly used to promote types of immunity not effectively generated by the nonadjuvanted antigens. A variety of compounds have been examined as adjuvants including mineral salts, emulsions, microparticles, saponins, microbial products, liposomes and cytokines (7,8). Based on their proposed mechanism of actions, adjuvants can be divided into two classes: delivery systems and immunostimulatory adjuvants (7,9). Delivery systems concentrate and present vaccine antigens to antigen presenting cells (APCs) and help colocalize antigens and immune potentiators to increase specific immune responses (7,10). Immunostimulants, such as cytokines or bacterial components, activate immunity directly or through pathogen recognition receptors (PRRs), respectively, to increase the immune responses to antigens (7). Despite the wide use of adjuvants in vaccines, the underlying mechanisms by which they potentiate immune responses are not well characterized. However, available evidence suggest that adjuvants induce immune responses by a combination of the following mechanisms: (i) activation, maturation and migration of APCs to the

draining lymph nodes, (ii) induction of cytokines and chemokines; (iii) enhancement of cellular recruitment of important immune modulating cells at the site of injection; (iv) increase antigen uptake and presentation to APC; (v) activation of PRRs and inflammasomes (11). Both delivery systems and immune potentiators serve to augment vaccine-induced Ag-specific responses *in vivo*. Clearly, since adjuvants can affect the immune responses in different manners, inducing humoral vs cellular immunity, well-informed and rational selection of adjuvants will contribute to development of effective new specific vaccine formulations.

Major Benefits of Adjuvants

Adjuvants can have a variety of other beneficial advantages besides the traditional role of being used to improve the immunogenicity of vaccine immune responses (**Figure 1.1**). First, adjuvants can enable the use of lower vaccine doses, greatly expanding the supply when large-scale vaccination is urgent in the case of a major pandemic outbreak of infection (12). Second, vaccines can benefit from suitable adjuvants by inducing a more rapid immune response and thus reducing the number of immunizations to achieve effectiveness (13-15). This approach would be beneficial for (i) vaccine delivery in parts of the world where compliance can be an issue and (ii) for the development of biodefense vaccines against potential bioterrorism weapons where a single-shot vaccine would be critical. A third advantage of adjuvants would be broadening the repertoire (breadth and/or specificity) of antibody responses, which could be crucial to the success of vaccines targeting pathogens that undergo antigenic drift or strain variation (16-18). Finally, a major role for the inclusion of adjuvants would be to aid in the development of new effective therapeutic T cell inducing vaccines. These are

practical applications by which adjuvants can be specifically used to improve future vaccines for unmet needs.

Importance for T Cell-Based Vaccine Adjuvants

Although widespread use of vaccines with adjuvants has had an extraordinary impact on global health, there remain many infectious and other diseases for which vaccines are not available. The difficult challenge remains in developing adjuvants that generate effective CD8⁺ T cell responses for chronic viral infections and for therapeutic treatment of cancers. Historically given that most vaccines confer protection through humoral immunity (19), it led to the development of adjuvants focused at inducing and/or enhancing antibody responses. As a consequence, a major limitation of the currently licensed vaccine adjuvants are ineffective at inducing CD4 T helper 1 (T_H1), especially CD8 T cell immune responses (20,21), which are required for either controlling or preventing the onset of chronic infections and cancer. Therefore, they are not optimal for many of the challenges in vaccination today. And given that there are still no effective vaccines against Tuberculosis (TB), Malaria, HIV, or for cancer therapy, developing adjuvants that can generate potent and durable protective T cell immunity will have a profound clinical impact for a variety of diseases. This underscores the critical need to develop vaccines with appropriate adjuvants capable of evoking the desired potent and durable Ag-specific CD8 T cell immunity.

CD8 T cells

The goal of successful vaccination is the induction of the potent CD8 memory T-cell populations to rapidly control infection or disease. CD8 T cells are an important component of the immune response to infection and cancer (22-25). CD8 T cells (as known as cytotoxic T lymphocyte, CTL, cytolytic T cell, Tc, or killer T cells) mediate their effector functions through production of cytokine such $\text{IFN}\gamma$ and $\text{TNF}\alpha$ and/or secretion of perforin or granzyme (23,26). After infection or immunization, Ag-specific CD8 T cells respond in 3 distinguishable phases: expansion, contraction and memory (23,27,28). When naïve CD8 T cells are primed they undergo clonal expansion when its TCR comes in contact with its cognate peptide-bound MHC class I molecule presented by APCs. After activation it culminates in a higher frequency of antigen-specific CD8 T cells that can enter the blood and migrate to the site infection inducing rapid effector function capable of killing infected cells. CD8 T cell activation is also dependent on a second signal, which is the engagement of the CD28 molecule on the T cell with the costimulatory molecule CD80 and CD86 expressed on APCs. In addition, simultaneous external influences, such as the presence of inflammation and cytokines (e.g. IL-2, IL-12, IL-7, IL-15) are also essential for T cells to undergo full T cell proliferation and differentiation into Ag-specific memory T cells (29-32) (**Figure 1.2**). After pathogen clearance, the Ag-specific CD8 T cells undergo contraction, where the bulk of the effector T cells die. However, a small number of effector cells survive, leading to established long-lived CD8 memory T cell subsets, which are the basis for protective immunity against infection and diseases (33). Thus, given that CTLs can survive long term in the absence of antigen and provide protection against recurrent infections (26,34,35), enhancing the quantity and quality of memory CD8 T cells is the ultimate

goal for improving the efficacy of most vaccines.

DNA Vaccines

In recent years, improved DNA vaccines have now reemerged as a promising lead candidate for therapeutic intervention due to their ability to potentially induce CD8 T cell immunity in humans (15,36-38).

The DNA vaccine platform first came into the spotlight in the early 1990s, when it was reported that the delivery of plasmid DNA into the muscle induced an immune response against encoded viral antigens (39-41). In 1993, Wang *et al.* were the first to show immune responses against a chronic viral infection (40). Subsequently, Margaret Liu *et al.* (39) and Harriet Robinson *et al.* (41) both independently reported that injecting plasmid DNA encoding influenza A nucleoprotein intramuscularly generated both humoral and cellular immune responses against influenza virus antigens in mice. Subsequently, David Weiner and colleagues at the University of Pennsylvania would show that DNA plasmids carrying HIV antigens engendered the induction of both Ag-specific antibodies and cytotoxic T lymphocytes (CTLs) (42). These findings introduced the potential of DNA as an immunization platform.

DNA vaccination has been suggested as an ideal therapeutic strategy due to numerous advantages over competing platforms. For example, DNA vaccines are non-live and non-replicating and thus unable to revert into virulent form, unlike live vaccines. Furthermore, DNA vaccines are highly customizable and hence, multiple antigens can be encoded within a single DNA plasmid. This allows for a much greater breadth in the host immune response and better protection as different epitopes can elicit different types of immune responses (43). In addition, individuals receiving DNA vaccines are unlikely to harbor anti-plasmid vector immunity, as seen with adenovirus vectors. For this

reason, DNA therapeutic vaccinations can be delivered repeatedly without initiating an immune response against the DNA plasmid (44,45). Moreover, DNA vaccines are simple and inexpensive to construct, can easily be produced in large quantities, are more temperature-stable than conventional vaccines, and can be easily stored and transported (45). Finally, DNA encoded antigens can be processed via both MHC class I and MHC class II pathways. Thus, DNA vaccines can drive the diverse induction of T_H1 , T_H2 and CD8 T cell responses, unlike recombinant protein vaccines that mainly drive T_H2 skewed responses. These advantages may help contribute to the successful delivery and administration of therapeutic vaccines to infected individuals in developing nations.

The success of DNA vaccines in preclinical studies quickly lead to clinical trials, and the idea of using DNA to immunize people immediately gained widespread recognition. The first DNA vaccine studies in humans were conducted over 15 years ago. The goals of the various studies were to evaluate and demonstrate the safety, tolerability and immune potency of the DNA vaccines. In the first Phase I clinical trial, a DNA vaccine for HIV-1 infection was evaluated for both therapeutic and prophylactic applications (46). Soon other DNA vaccine trials would follow, including trials that tested DNA-based vaccines against other HIV antigens, HBV, and malaria (47-49). These introductory studies established that DNA vaccines were tolerable in humans, and that they could enhance T cell proliferation and CTL activity (50,51), although the immune responses elicited were weaker than expected based on preclinical data. Although 'first-generation' DNA vaccines failed to demonstrate a robust level of vaccine-specific immunity in humans, extensive research continued to develop new modifications and improvements to the technology to enhance DNA efficacy.

To date, a plethora of approaches have been conducted to significantly improve the immunogenicity elicited by DNA vaccines, and as a result has sparked great

excitement and interest in the DNA platforms to be examined for therapeutic approaches and vaccines for unmet needs. These efforts have included optimization of the vaccine vectors (e.g. RNA/codon optimization) and antigens encoded by the plasmids (e.g. consensus sequences) to enhance antigen expression and cellular/humoral cross-reactivity (45,52); and *in vivo* electroporation (EP), a promising delivery method that improves the expression and presentation of antigens expressed by DNA vectors (45). EP is a simple, direct approach that involves the application of short electrical pulses to the vaccine delivery site. This transiently increases cell membrane permeability allowing for increased plasmid uptake and increased expression in the target tissue of mice, pigs and rhesus macaques (45,53-56). Although the mechanism for DNA delivery by EP is not fully understood, the procedure has improved plasmid transfection efficacy by a factor of 100 fold or greater and as result, has increased immunogenicity of DNA vaccines in both small (mice, guinea pigs, and rabbits) and large animal models (pigs, rhesus macaques, and chimpanzees) (56-64). DNA vaccination in combination with EP is a novel, safe, and effective strategy that elicits a strong, broad, and long-lasting humoral and cellular immune responses. EP is advantageous as a vaccine delivery approach because it can broadly activate CD8 cytotoxic T cells (CTL) that eliminate cells infected with intracellular pathogens. Thus, EP has been successfully used to enhance both cellular and humoral responses in small animal models and humans (55,56,65,66). More recently, Bagarazzi M.L., *et al.* (38), have published exciting data on EP administration with a DNA-HPV vaccine that opens many exciting new avenues for this combined technology approach to treat or prevent against many human pathogens. Bagarazzi and colleagues (38) reported that a therapeutic DNA vaccine co-administered with EP on its own in humans could produce long-lived CD8 T cells with cytolytic activity. The results also show that DNA delivered by EP is safe and tolerable (38). Finally, the

inclusion of molecular cytokine adjuvants to enhance, modulate and skew a desired immune response has become a promising way for significantly enhancing the efficacy of the DNA vaccine platform.

Cytokines

The human immune system is constantly in a perennial battle against infectious agents that cause disease and often, death. Therefore, to resolve such conflicts the immune system has developed countless strategies to effectively vanquish pathogenic intruders. One essential tactic is the release of chemical messengers known as cytokines. Cytokines are a group of proteins that promote copious biological functions that help regulate and generate the immune system (e.g. inducing maturation of APCs, differentiation of T_H1 and T_H2 cells, and inducing NK cells and CTLs) (67). Cytokine activity is highly pleiotropic as many cells can produce one to several cytokines therefore influencing many phenotypic traits of immune cells (68,69). They facilitate inflammatory and proliferative responses, differentiation, and crosstalk between immune cells; cells which perform the real-time fight against harmful pathogens that enter the body (68,70). Because of their crucial role in triggering the innate and adaptive immune responses for fighting off infections, certain cytokines, such as IL-2, $TNF\alpha$, IL-15 and GM-CSF, have been used as vaccine adjuvants to increase memory response against some infectious diseases and cancer (68,71,72). Therefore, cytokines are promising vaccine adjuvants for boosting the immune responses for the prevention, control, and treatment against infectious pathogens and cancers.

DNA Co-delivery with Molecular Adjuvants

One important approach with regard to increasing the potency of DNA based vaccines is the ability to manipulate the immune response through co-administration of cytokine genes. Genetic molecular adjuvants are normally administered as plasmids encoding a specific cytokine, chemokine, or costimulatory molecule. Indeed, the addition of immune modulatory adjuvants as part of a vaccine cocktail has been demonstrated to boost the adaptive immune response (73). A number of groups have shown that cell-mediated responses can be modulated both quantitatively and qualitatively through co-immunization with cytokine-expressing plasmids (**Table 1.2**). Specifically, it was demonstrated that co-immunization with T_H1 type cytokines (e.g. IL-12, IL-18) can enhance cellular immunity and bias the immune response toward a T_H1 type response, while T_H2 -type (e.g. IL-4) cytokines can boost antibodies responses and promote a T_H2 -type bias immune response (74,75). In choosing an adjuvant that provides a T_H1 or T_H2 biased response, it is important to consider which type of response may be more helpful in contributing to protection. For example, Tuberculosis (TB) and *Leishmania major* require a T_H1 -type response for effective immunity, while other parasitic and microbial infections require a T_H2 -type response (76-78). This ability to modulate or enhance the immune response in a defined manner has great promise to improve vaccine design and development.

The exact mechanism by which DNA molecular adjuvants induces an immune response is not fully understood. However, it is assumed first that after injection of DNA plasmids encoding the antigen of interest and cytokine adjuvant, local APCs, myocytes, monocytes or keratinocytes are transfected (45). Once cells are transfected, the plasmid encoded antigens and cytokine genes are expressed. In the case of the antigen, it is processed, and peptides are presented in the context of Class I or Class II MHC

molecules by DCs, affording these cells to stimulate CD4 and CD8 T cells in the secondary lymphoid organs (**Figure 1.3**). Meanwhile, as the antigen presentation by DCs is perpetuating an immune response, the transfected cells are subsequently producing the selected cytokine to help shape and augment the desired immune responses (**Figure 1.3**).

The inclusion of different cytokines is actively being studied as a way to induce and shape both innate and adaptive immune responses. For example, one of the most studied and tested cytokine adjuvants has been IL-12. IL-12 a cytokine produced by DCs supports the differentiation of Ag-specific CD4 T cells to produce T_H1 cytokines as well as prompts the expansion of Ag-specific CD8 T cells to express cytotoxic molecules, such as granzyme B, perforin, and IFN γ (79). Kim J.J. et al. were the first to investigate the role of co-delivery of IL-12 with DNA vaccines, observing an increase in specific CTL responses when mice were coimmunized with a HIV-1 DNA vaccine plus an IL-12 plasmid (81). Moreover, IL-28, a cytokine that belongs to the Interferon III/lambda (IFN γ) family cytokines has also been shown to play a role in the adaptive immune response (82,83). Its inclusion as an immunoadjuvant during small animal and NHP vaccination led to augmented Ag-specific T_H1-biased responses, as well as an increased cytotoxic potential in CD8 T cells (84-86). Finally, granulocyte macrophage colony-stimulating factor (GM-CSF), a cytokine secreted white blood cell growth factor has also been used a molecular adjuvant and has been shown to enhance both cellular and humoral responses in mice and non-human primates (87-90). This indicated that our search for improving effective vaccine-induced CD8 T cell responses for diseases where vaccines are still needed such as HIV, TB, malaria and even cancer, may lie in the discovery of novel molecular cytokine adjuvants.

While certain immunoadjuvants have been shown to enhance the potency of T_H1 vaccine-induced responses, it has been a challenge to find adjuvants that can enhance polyfunctional CD8 T cell responses. Every year new insights into the biological functions of cytokines or cytokine-like molecules are being discovered, with many still waiting to be discovered. However, recently, two novel immunomodulatory molecules, IL-33 and ISG15, have been demonstrated to drive Th1-type cell-mediated viral and/or tumor immunity (91-93). Therefore, this led us to explore the use of Interleukin 33 and Interferon stimulating gene 15 as effective vaccine adjuvants to boost a T_H1 and CTL immune responses, a desired result for effective vaccination against cancer and chronic infections.

Interleukin 33 (IL-33)

Interleukin 33 (IL-33) was first described in 1999 by Onda H and colleagues who identified it as DVS27—a 30-kDa protein highly expressed in canine vasospastic cerebral cells (94). Six years later, through computational sequence comparison, Schmitz and colleagues revealed that the C-terminal end of IL-33 contained a β -sheet trefoil-fold structure characteristic of the Interleukin 1 (IL-1) family (95). IL-33 then became the 11th identified IL-1 family member. Subsequently, IL-33 was recognized as the functional ligand for the orphan IL-1 receptor ST2 (also called IL-1R-like-1) (95). ST2 is selectively expressed on the cell surface of T_H2 cells and not on T_H1 cells (95). Therefore, IL-33 has been studied primarily for its role in the context of T_H2 immunity and T_H2 -related diseases such as asthma, atopic dermatitis, and anaphylaxis (95,97,98). However, recent studies are beginning to show that IL-33 cytokine activities far exceed the realm of T_H2 immunity by promoting T_H1 immune responses and influencing the development of antiviral CD8 T cells (**Figure 1.4**) (91).

While historically isolated from keratinocytes, epithelial cells, and endothelial cells, IL-33 is now known to be released from a variety of tissue types as a pro-inflammatory cytokine (97-99). Specifically, IL-33 functions as an alarmin by signaling tissue damage to local immune cells after exposure to pathogens, tissue damage, or death by necrosis (**Figure 1.5**) (99). IL-33 is predominantly expressed at the epithelial barrier as the first line of defense against pathogenic threats. Through its cognate receptor ST2, IL-33 activates a variety of cells: hematopoietic cells, mast cells, eosinophils, basophils, Natural Killer (NK) cells, Natural Killer T (NKT) cells, CD8 T cells, T_H2 lymphocytes, and non-hematopoietic cells (99). In addition, IL-33 signaling can be negatively regulated by a soluble form of ST2 that lacks transmembrane domain, which behaves as a decoy receptor (97,98,103) (**Figure 1.5**).

IL-33 exists in two biologically active isoforms: full-length IL-33 (proIL-33) and mature IL-33 (mtrIL-33). These two isoforms can operate in at least two spaces—nuclear and extracellular (100,101), highlighting IL-33 dual-function cytokine properties. The nuclear space is the exclusive domain of proIL-33 and it is able to concentrate there via its amino terminus that contains a non-classical nuclear-localization sequence and a short chromatin-binding motif (99). This is where IL-33 is usually found; however, when released by inflammation or stimulation, proIL-33 is often digested into mtrIL-33, a form with a lower molecular weight (18-kDa). Unlike proIL-33, mtrIL-33 is not capable of localizing into the nucleus because it lacks the N-terminal nuclear-localization sequence. Currently, the nuclear function of proIL-33 is unclear, but recent studies have suggested it may play a role in transcriptional repression and chromatin compaction (100,102). Extracellular proIL-33 and mtrIL-33, on the other hand, are known to bind to their cognate receptor ST2, activating the MyD88-signaling pathway which induces the production of various cytokines and chemokines or causes cell differentiation,

polarization, and activation, depending on the target cell (**Figure 1.5**) (103,104). While one might assume that they induce similar effects because they bind to the same ST2L, Luzina *et al.* demonstrated that proIL-33 can promote inflammation differently from mtrIL-33 in an ST2-independent fashion (**Figure 1.5**) (105). This study showed that compared to proIL-33, mtrIL-33 produced a strong T_H2-skewing cytokine profile (105). However, the processing and release of IL-33 appears cell-type specific and how proIL-33 is digested into mtrIL-33 is still a matter of debate (99).

IL-33's Role in Antiviral and Antibacterial Immunity

Multiple groups have shown that IL-33 activity is primarily associated in driving T_H2-immune responses, particularly in augmenting cytokine levels of IL-4, IL-5 and IL-13 (98). However, it is now beginning to surface that IL-33 has functions that surpass T_H2 immunity as it can contribute to the development of T_H1-type immune responses and promote CD8⁺ T cell responses (99).

Given its ability to direct these T_H1-type immune responses, it is reasonable to suggest IL-33 may also be essential in inducing protective immunity against viral infections. Some of the 1st studies to implicate IL-33's pro-T_H1 cytokine activities observed its biological target on NKT cells (106,107). These studies showed that exposure to IL-33 privileged the production of IFN γ by NKT cells in response to TCR engagement and in the presence of IL-12. More recently, several studies have shown that this activity was not restricted to NKT cells. Yang *et al.* showed that CD8⁺ T cells can also express ST2 and respond to IL-33 (108). They reconfirmed the notion that IL-33 synergizes with TCR and/or IL-12 signaling to augment IFN γ production in effector CD8 T cells (108). Consistent with these findings, Bonilla *et al.* showed that following LCMV

infection in mice, roughly 20% of activated Ag-specific CD8 T cells expressed ST2 (91). Subsequently, Sesti-Costa et al, demonstrated in a Coxsackie virus infection model, that mice treated with recombinant IL-33 (rIL-33) increased IFN γ and TNF α secretion by CD8 T cells and NK cells, which correlated with viral clearance of Coxsackie virus (109). These studies provide evidence that IL-33 can drive protective antiviral CD8 T cell responses *in vitro and in vivo*.

On the hand, studies on IL-33's protective role against bacterial infections have been limited and are only beginning to be appreciated. Li *et al* showed in a mouse model of *S. aureus* skin infection, delivery of rIL-33 improved antibacterial defense by activation of nitric oxide in macrophages (110). Moreover, a recent study by Lee *et al.* reported a positive correlation between IL-33 and IFN γ levels in patients with TB pleurisy (111). Clearly, IL-33 likely has protective antibacterial properties, however, more studies are needed to understand its role during bacterial infections. Together, these studies give insight into IL-33's new biological activity to direct T_H1 and effector CD8 T cells and its essential role in driving protective immunity against viral and bacterial pathogens.

IL-33's Role in Antitumor Immunity

There is a plethora of studies about the pleiotropic cytokine activities of IL-33 and its role in inflammation and its association with allergy and autoimmune diseases (97,98). However, its role in antitumor immunity and antitumor growth is only beginning to surface. Several recent studies have highlighted the important role of IL-33 in experimental mouse tumor models and have shown that IL-33 can drive antitumor CD8 T cell responses. Gao *et al.* used B16 melanoma and Lewis lung carcinoma (LLC) metastatic models to show that transgenic expression of IL-33 inhibited tumor growth

and metastasis in mice (92). Transgenic expression of IL-33 and delivery of recombinant IL-33 increased the infiltration of CD8 T cells and NK cells into the tumor and also increased their cytotoxicity both *in vitro* and *in vivo* (92). This study provides further evidence that IL-33 promoted the proliferation and activation of CD8 T cells and NK cells by activating the intracellular molecule nuclear factor- κ b (NF- κ b) and suggests a mechanism by which IL-33 might promote CD8 and NK activation and expansion. In addition, Gao *et. al.* recently also reported that tumoral expression of IL-33 can inhibit tumor growth and modify the tumor microenvironment by promoting the function of CD8 T and NK Cells (112). Interestingly, recent reports have reported that increase levels of IL-33 in human cancers may have a correlation with better diseases prognosis in HPV cervical cancer (113) or associated with hepatocellular carcinoma prolonged patient survival (114). Together, these studies demonstrate the notion that IL-33 increases the formation of Ag-specific CD8 T cells and reveals that IL-33 has immunotherapeutic implications in driving immune responses against cancer.

Interferon Stimulated Gene 15 (ISG15)

Interferon stimulated gene 15 (ISG15) was identified in 1984 by Ernest Knight Jr and colleagues as a 15kDa protein induced by type 1 interferons (IFNs) (115). Three years later, Haas and colleagues, showed a resemblance between ISG15 and ubiquitin (116). The crystal structure of ISG15 showed its similarity to ubiquitin by revealing that ISG15 consisted of two ubiquitin-like domains, located at its N-terminal and C-terminal ends of the protein (117). ISG15 is synthesized as a 17kDa precursor that is cleaved at its C-terminal domain to yield the mature form of ISG15 (118). Unlike ubiquitin which is highly conserved, ISG15 varies between species (119). However, similar to ubiquitin, the

mature form contains the ubiquitin-like C-terminal LRLRGG motif that is essential for covalently attaching itself to the lysine residues of target proteins by an E1-E2-E3 enzyme process called ISGylation (**Figure 1.6**) (117). This enzymatic cascade that is similar to, yet distinct from that of ubiquitin conjugation has been studied in great detail and has been reviewed elsewhere (93,119-121). Several proteomic strategies have been conducted to catalogue ISGylated proteins, identifying more than 300 candidates (122). However, how ISGylation affects their cellular distribution and function remains to be elucidated (123). ISG15 is one of the most highly induced transcripts after type 1 IFNs stimulation or other stimuli, such as exposure to viral infections, lipopolysaccharides, and TNF α stimulation (93). These types of stimuli activate transcription factors in IFNs signaling, mainly IRF3 and ISGF3 (119-123), which in turn upregulate expression of ISG15 (**Figure 1.6**). For instance, in Type I IFN receptor R1 knockout mice, ISG15 production significantly decreases upon treatment with Gram-negative bacteria lipopolysaccharide (LPS) or viral infections (93), suggesting that induction of Type I IFN secretion triggers ISG15 expression. Therefore, ISG15 is essential for the control of certain viral and bacterial infections.

Antiviral and Antibacterial Activity of ISG15

First, given that IFNs play an important role during viral infections, and second, that ISG15 and its ISGylation are strongly induced by Type 1 IFNs, many studies have explored their contribution to antiviral activities (93,125). Briefly, several *in vitro* challenges have shown that ISG15 can regulate viral growth or titers of Dengue, West Nile, HIV, Ebola, Japanese encephalitis and Influenza A virus (93). For instance, the delivery of small interfering RNA knockdown of ISG15 in wild-type murine cells or human airway epithelia cells were found to increase the titers of dengue virus and influenza A

viral titers, respectively (93). Moreover, increase lethality has been observed in ISG15^{-/-} mice after infection with Influenza A and B viruses, herpes simplex virus type 1, Sindbis virus and Chikungunya (93). ISGylation does not usually cause substrate degradation like ubiquitin (119,123), but can function to regulate protein levels, signaling pathways, vesicular trafficking, and numerous other undefined regulative roles (119). While, it is known that ISG15 conjugates to hundreds of target proteins, how ISG15 conjugation mediates a broad range of protection still remains to be fully elucidated (93,126). Although a number of studies have shown ISG15 antiviral protective properties, no studies have evaluated ISG15's biological role during bacterial infection or its protection *in vivo*. ISG15 mediated protection against bacterial infection is largely unknown and future studies are warranted.

Antitumor Activity of ISG15

ISG15 has also been implicated in host defense pathways that serve antitumor functions. To initiate the process of ISG15 conjugation (ISGylation), ISG15 must first utilize the E1 enzyme (UBE1L) (**Figure 1.6**) (93). Without this enzyme ISGylation of specific target proteins cannot occur. UBE1L has been demonstrated not to be detected in all human lung cancer cells lines (122-124), implicating a tumor-suppressive role of ISGylation and suggesting the potential role of ISG15 in facilitating tumor control or immunity. Human leukemia cells have also been found to lack functional E1 enzymes and inducing ectopic expression of UBE1L restore ISGylation and enhanced IFN signaling (127). Moreover, ISG15 has also been reported to be induced during cancer chemotherapies, as studies have shown that ISG15 was upregulated following camptothecin treatment of colorectal and breast cancer cells (122-124). Collectively, these studies potentially demonstrate that reduced ISGylation may be linked to a

malignant phenotype, and enhanced ISGylation correlated with the therapeutic response to anticancer signals. In contrast, a few studies have also demonstrated that overexpression of ISG15 or ISGylation positively correlated with tumorigenesis (122-124). This relationship is complex and a universal consensus on the relationships between ISG15 and tumor development is still missing. A similar yin and yang role in human cancers have also been reported for other cytokine mediators such as IL-2 and HGMB1 (128,129). A better understanding of the potential function of ISG15 in tumor immunity or in the tumor microenvironment requires future studies. Nevertheless, given ISG15's ability to be induced by a diverse stress of stimuli, suggests that it may serve broader functions beyond the innate immune response.

Free ISG15 Modulates Immune Responses

In addition to its conjugated form, ISG15 can also exist in a free unconjugated form both intracellularly and extracellularly (**Figure 1.6**). Both forms of free ISG15 could influence cellular functions. For instance, intracellular free ISG15 has been shown to control the ability of Ebola VLPs to egress from cells *in vitro* (93,130). Over expression of ISG15, independent of the conjugation cascade, consequently decrease Ebola VP40 VLP release (93,130). More interestingly, the secreted ISG15 form can act as cytokine and modulate the immune responses (125). ISG15 is synthesized in many cells and has been shown to be secreted by monocytes, lymphocytes and neutrophils *in vitro* (126). The underlying mechanism of how ISG15 is secreted from the cell remains unknown. The secretion of free ISG15 was first described in the early 90s by Ernest Knight Jr and Beverly Cordova demonstrating secretion of ISG15 from human lymphocytes and monocytes (131). Subsequently, Ernest Knight Jr and colleagues would report on its function to induce secretion of IFN γ from peripheral blood mononuclear cells (PBMCs)

(132). Five years later, in 1996, Ernest C. Borden and colleagues would confirm the immunoregulatory properties of ISG15 to induce IFN γ production from PBMCs (133). Although given these exciting results of ISG15 cytokine-like properties, no further studies on free ISG15 would be made for almost two decades. In 2012, Casanova and colleagues would reconfirm the cytokine-like role for ISG15 by demonstrating that ISG15-deficiency was associated with a loss of IFN γ , which in turn led to increased susceptibility to mycobacterial disease in both mice and humans (134). They reestablished that ISG15 can induce IFN γ production in PBMCs and also demonstrated that NK cells upregulate IFN γ production in response to recombinant ISG15. These collected studies support the ability of free ISG15 to function as an immunomodulatory molecule to regulate IFN γ production and that ISG15 contributes to host protection against infectious disease through both conjugation-dependent and –independent modes of action. Therefore, given that ISG15 has been implicated in the regulation of IFN γ and NK cells, both which are important mediators of viral and tumor immunity, it is conceivable that ISG15 could be used as a vaccine adjuvant to help fight pathogenic infections or even cancer. The mechanism by which all three forms of ISG15 exert antiviral or antitumor effects is an important area of research that remains poorly understood.

Goals of this Thesis

CD8 T cells play a crucial role in mediating protection against cancers and to a variety of chronic infections, including HIV, tuberculosis (TB) and malaria. An overall shortcoming of currently licensed vaccines, especially non-live vaccines, is their inability to generate effective CD8 T cell responses. One way to improve the quantity and quality of immune responses during vaccination is to incorporate adjuvants, which have been shown to help increase their immune potency. However, it has been a challenge to discover adjuvants that can amplify the induction of CD8 T cell responses. This highlights the need to develop new vaccine adjuvants capable of inducing potent and durable CD8 T cell immunity in human-kind.

Different vaccine platforms have been studied, but the development of DNA-based vaccines in conjunction with immunomodulatory 'cytokine' adjuvants has emerged as particularly promising for inducing Ag-specific CD8 T cell-mediated immune responses (15). Recently, two immunomodulatory molecules have been demonstrated to play a role in inducing T_H1-type cell-mediated immunity: cytokine IL-33 and cytokine-like molecule ISG15 (91-93). Therefore, in this thesis, I tested the hypothesis that both IL-33 and ISG15 delivered as molecular adjuvants can increase the CD8 T cell potency of DNA vaccines. Specifically, I proposed that IL-33 and ISG15 can act as vaccine adjuvants to enhance the CD8 T cell immunity against intracellular chronic pathogens and tumors. I used different disease models to study the efficacy of IL-33 and ISG15 as DNA vaccine adjuvants. Additionally, this thesis aims to give further insight into the biological function of IL-33 and ISG15 to modulate the cell-mediated immune responses. Overall, I believe that identifying new molecular adjuvants that elicit effective vaccine-induced CD8 T cell immunity may be critical for the elimination of many challenging diseases including TB, HIV, malaria and cancers.

FIGURES

Table 1.1. Licensed adjuvants in humans

Adjuvants	Immunostimulatory component(s)	Immune response activated	Licensed vaccines
Alum	Aluminum salts	↑ Ab responses ↑ Th2 responses	DTaP, Hepatitis A, HepB, pneumococcus, HPV (Gardasil)
MF59	Squalene-in-water emulsions	Balanced Th1 + Th2 responses	Influenza (Fluad®), H5N1 pandemic (Aflunov®), H1N1 pandemic (Focetria®)
AS03	Squalene-in-water emulsions	↑ Ab responses	Flu pandemic (Pandemrix®)
AS04	MPL plus alum	↑ Ab and Th1 responses	HPV (Cervarix) HepB (Fendrix)

Ab, antibodies; MPL, monophosphoryl lipid A; Th1, CD4 T helper 1; Th2, CD4 T helper 2

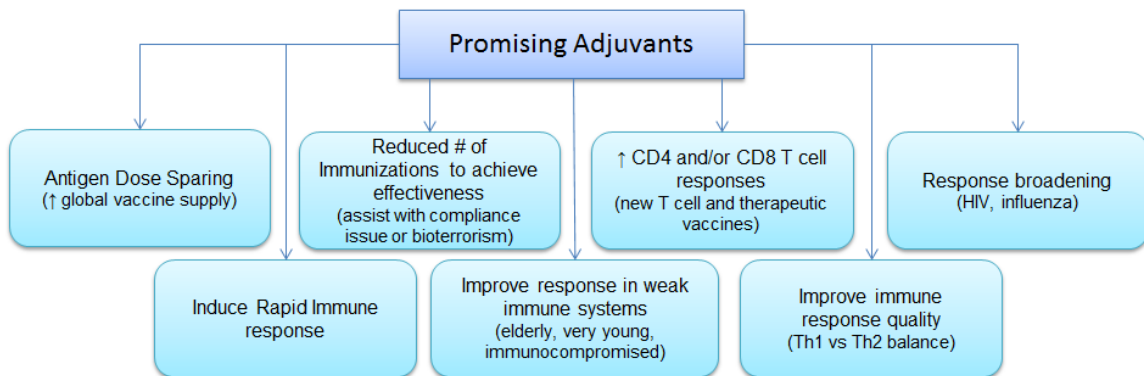


Figure 1.1. Major benefits of adjuvants. These are all factors by which idea adjuvants can be used to improve future vaccines against many challenging diseases where no effective vaccines still exist.

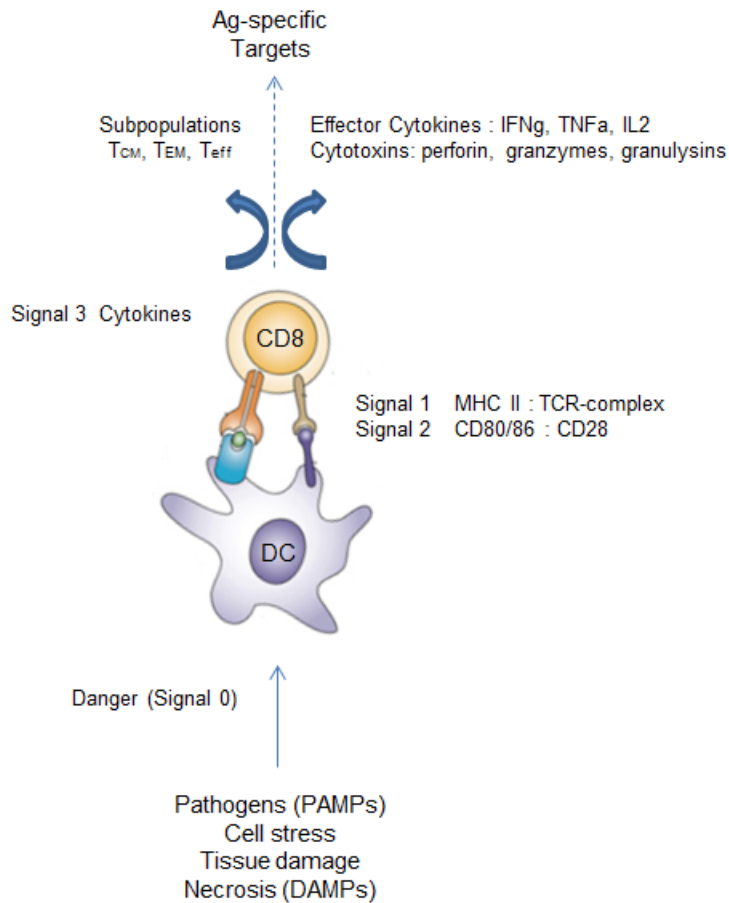


Figure 1.2. CD8 T cell priming and activation. When innate cells interact with pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) or some inducible signal, the immune system is initiated (Signal 0). Dendritic cells uptake protein antigen, process it into smaller peptide fragments and load them onto MHC Class I or II molecules. These mature DCs with highly upregulated costimulatory molecules due to signal 0, then migrate to the draining lymph nodes where their MHC-peptide complex can be recognized by the T cells expressing the matching cognate T cell receptor (TCR) (Signal 1). For proper activation of CD8 T cells they require a second signal, provided by binding to costimulatory molecules CD80 and CD86 on APC by CD28 (Signal 2). As a result, CD8 T cells secrete effector cytokines and cytotoxins, which can directly kill pathogen infected cells or tumor cells. In addition, proinflammatory cytokines are also essential for proper activation, differentiation and expansion of memory CD8 T cell subsets or can further amplify costimulatory molecules (Signal 3).

Table 1.2. Molecular cytokine adjuvants codelivered with DNA vaccines

Cytokine Adjuvants	Antigens	References
IL-2	HIV	73,88
IL-7	HSV, HCV	135
IL-12	HIV, HBV, HSV	73-75,81,87,136-138
IL-15	HIV, HSV	73,75,139,140
IL-18	HIV, HSV	137,141,142
IL-21	HSV	143
IL-23	HCV	144
IL-28	HIV	84-86
GM-CSF	HCV, HIV	144

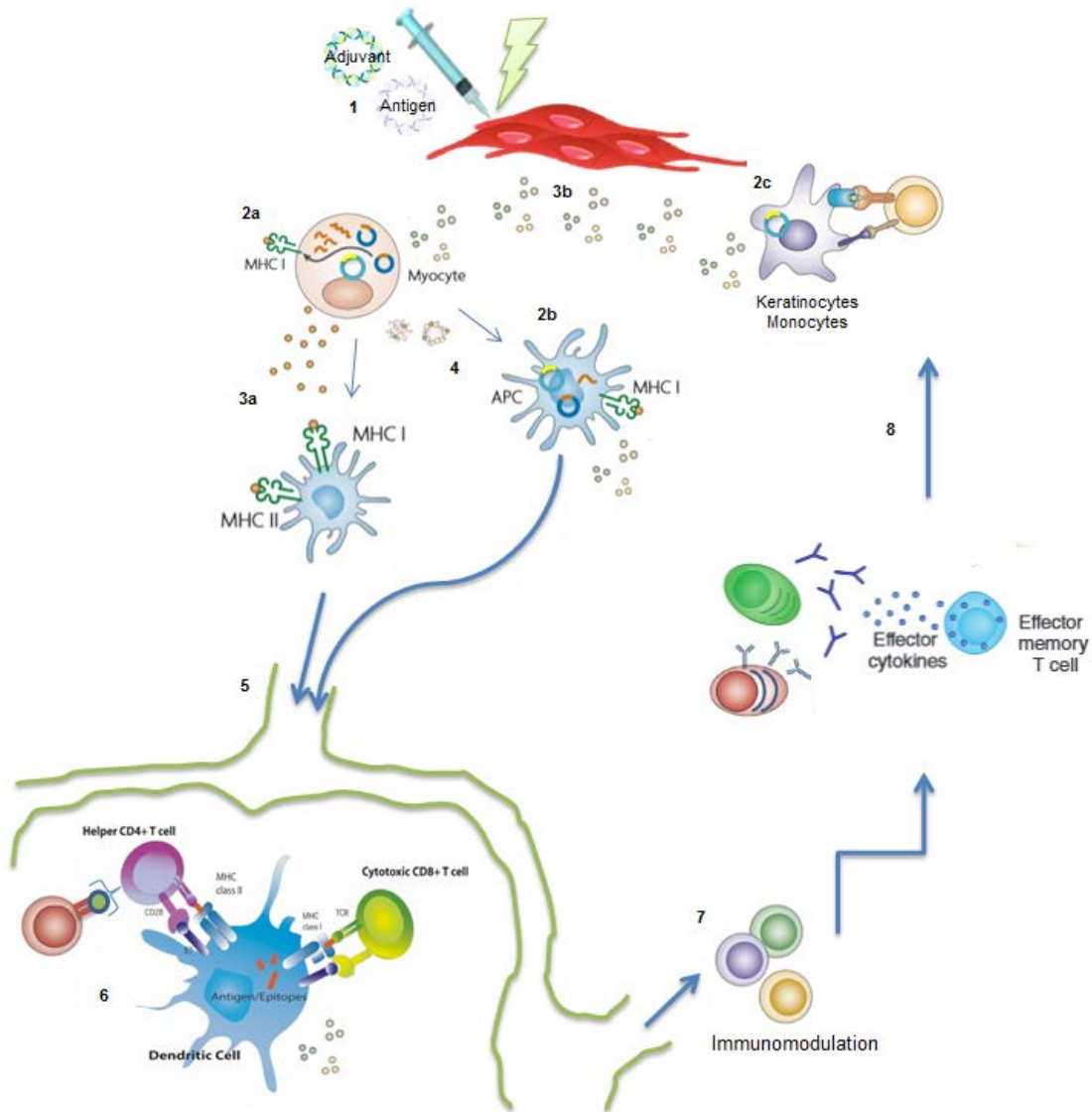


Figure 1.3. Proposed mechanism of DNA vaccination with molecular adjuvants. (1) Vaccine formulation containing two separate DNA plasmids, one that contains the antigen target and the other that contains the immune adjuvant gene, is administered in the muscle followed with electroporation. After immunization, myocytes (2a), resident antigen presenting cells (APCs) (2b), and other cells (e.g. keratinocytes, monocytes) (2c), are transfected with the plasmids, leading to protein and cytokine production. The protein processed and/or released by transfected cells are presented in the context of MHC class II (3a) or MHC class I (2b) molecules which stimulate CD4 and CD8 T cells, respectively. Peptides can also be presented on MHC class I by cross-presentation due to APCs engulfing apoptotic or necrotic bodies (4). Meanwhile, the transfected cells are simultaneously secreting the gene (e.g. cytokines or chemokines) adjuvant which in turn facilitates the recruitment of many various immune cells to the site. All these events lead more effective maturation and activation of immune cells. Mature antigen-loaded APCs then migrate to the draining lymph nodes (5) to interact with antigen-specific B and T cells (6) to activate both B (antibody secreting) and T cell (effector CD8) responses (7). These activated lymphocytes can now migrate to the inflammatory sites to provide protection against infectious diseases (8). At the infection site these activated lymphocytes could be restimulated and further expanded at the site of immunization by transfected cells (2c). In addition, the secretion of the selected adjuvant by transfected cell at site can lead to further enhancement of immunological parameters, perpetuating proper expansion and differentiation of memory T cells, thus leading to the proper establishment of immunological memory. Therefore, upon reinfection the established surveillance memory system could rapidly and effectively respond.

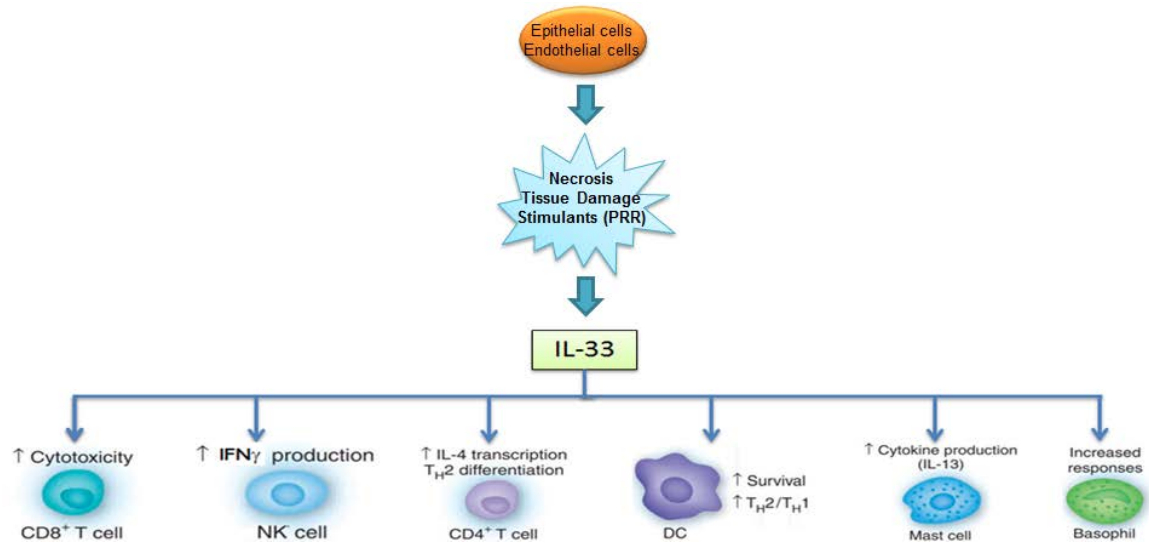


Figure 1.4. Interleukin 33 effects on innate and adaptive immune cells. Interleukin 33 (IL-33) can be secreted by neutrophils, macrophages, DCs, fibroblasts, but it is mainly secreted by epithelial and endothelial cells and is released in response to external stimuli or passively secreted due to tissue damage or cell neurosis. IL-33 has pleiotropic activities that act on a variety of innate and adaptive immune cells. The key effects they have on some of these cell types are indicated. DC, dendritic cell; IFN γ , interferon-gamma; NKT, natural killer T; T $_H$ 2, T helper 2; T $_H$ 1, T helper 1

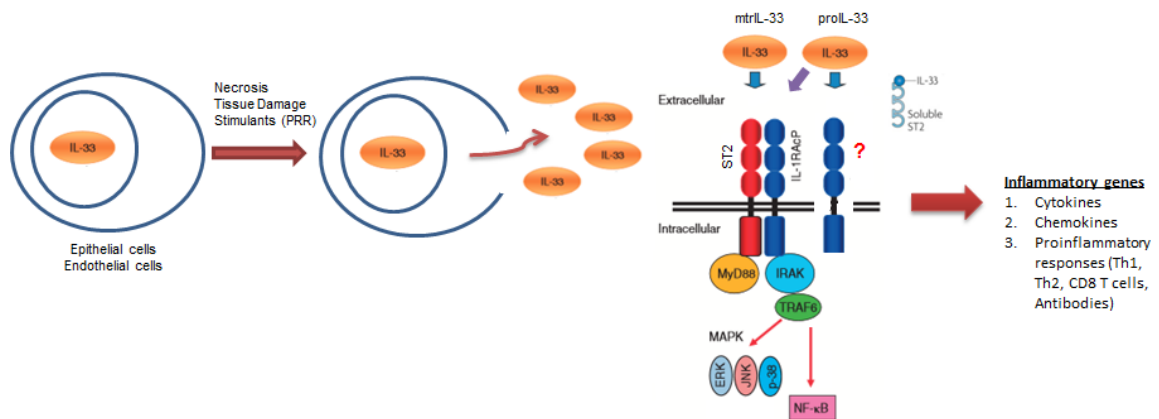


Figure 1.5. The basic biology of interleukin 33. In its normal state interleukin 33 (IL-33) resides in the nucleus of cells. Tissue damage or some inducible signal leads to the release of IL-33 from cells such as fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells. IL-33 can be released or secreted as two biological active forms: full-length IL-33 (proIL-33) and/or as processed proIL-33 into mature IL-33 (mtrIL-33). As a cytokine-alarmin, IL-33 signals through a heterodimer complex which consists of ST2 and IL-1 receptor accessory protein (IL-1RAcP). The binding of IL-33 to its cognate receptor results in recruitment of MyD88 and TRAF6, which then leads to the activation of the transcription factor nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. This signaling perpetuates an immune response, inducing gene expression which leads to cytokine and chemokine synthesis. Through a poorly defined mechanism proIL-33 can induce immune responses independent of its ST2 receptor. In addition, ProIL-33 not only acts as a secreted cytokine, but can also act as a nuclear binding factor. ProIL-33, which contains a nuclear localization and chromatin binding motif at its N-terminus, can migrate into the nucleus, and bind to acidic residues of dimeric histones (H2A-H2B). However, proIL-33's role in the nucleus is still not fully understood. Moreover, soluble ST2 can act as a decoy receptor to negatively regulate IL-33 signaling.

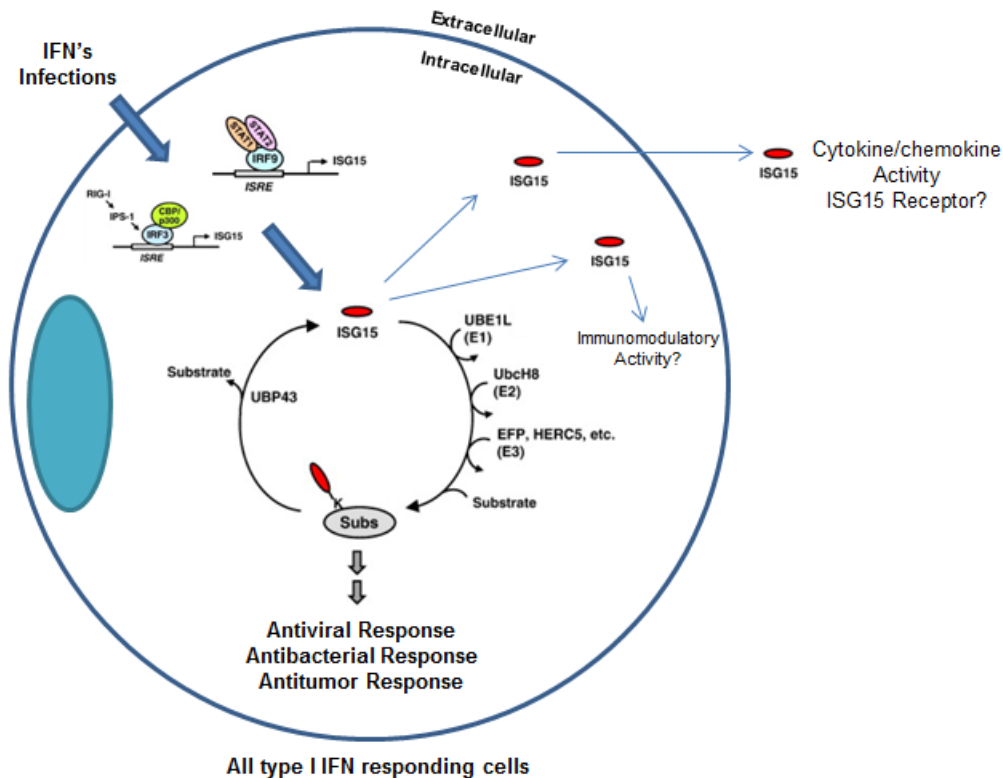


Figure 1.6. The ISGylation machinery and schematic roles of ISG15. ISG15 is strongly induced by type I interferons (IFNs), which initiates several initial signaling pathways to induce expression of ISG15. The first step, ISG15 is activated by UBE1L enzyme (E1) in an ATP-dependent manner. ISG15 is then transferred to UbcH8 (E2) and subsequently conjugates to target substrate proteins through the aid of E3 ligases (HERC5, EFP, HHAR1). The isopeptidase USP18 (deconjugating enzyme) reverses the coupling by removing ISG15 from target proteins. The ISGylation pathway can therefore be recycled. However, ISG15 can also exist as free intracellular ISG15 or as secreted extracellular free ISG15. The biological role of free intracellular ISG15 remains unknown. Secreted free ISG15 is known to have immunomodulatory properties, however, how it is secreted or how cells may respond to ISG15 (may bind to unknown receptors) remains to be determined. IFN, interferon; ISG, IFN-stimulated gene. Figure was adapted from Joen YJ, et al 2010.

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CHAPTER 2

ALARMIN INTERLEUKIN 33 ACTS AS A VACCINE ADJUVANT INDUCING POTENT ANTIGEN-SPECIFIC TUMOR IMMUNITY

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*“Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference”
-Robert Frost*

ABSTRACT

Interleukin 33 (IL-33) has emerged as a cytokine that can exhibit pleiotropic properties. Here we examine IL-33 for its immunoadjuvant effects in an HPV-associated cancer immune therapy model in which cell-mediated immunity is critical for protection. It is known that two biologically active forms of IL-33 exist: full-length IL-33 and mature IL-33. The potential ability of both isoforms to influence the adaptive immune responses and act as a vaccine adjuvant has not been well defined. We show that both isoforms of IL-33 are capable of enhancing potent antigen specific effector and memory T cell immunity *in vivo* in a DNA vaccine setting. We also show that while both forms of IL-33 drove robust IFN γ responses, neither form drove high secretion of IL-4 or any elevation of IgE levels. Further, both isoforms augmented vaccine-induced polyfunctional CD4 and CD8 T cell responses, with a large proportion of CD8 T cells undergoing cytolytic plurifunctional degranulation. Therapeutic studies indicated that vaccination either IL-33 isoform in conjunction with an HPV DNA vaccine caused rapid and complete regression of established tumors *in vivo*. Moreover, we show that IL-33 can significantly expand the magnitude of antigen-specific CD8 T cell responses and elicit effector-memory CD8 T cells. Overall, our results support the development of these two IL-33 isoforms as immunoadjuvant candidates in future vaccination against pathogens and in the context of anti-tumor immune-based therapy.

INTRODUCTION

Adjuvants are critical components of most clinical vaccines and are used to enhance adaptive immune responses to antigen (1). Adjuvants can help shape the quantity and quality of immune responses (1). However, currently available FDA-licensed adjuvants are poor inducers of CD4⁺ T helper 1 (T_H1) and even worse at treating CD8 T cell responses (2,3). It is important to identify a new generation of potent vaccine adjuvant(s) that can drive and specifically direct these desired responses. Thus, the inclusion of different molecular adjuvants, such as cytokines, is actively being studied as a way to increase the efficacy of vaccines. Different vaccine platforms have been studied, but the development of DNA-based vaccines in conjunction with cytokine adjuvants, has emerged as a particularly promising for inducing anti-viral and anti-tumor cell-mediated immune responses (4,5). Indeed, the potency of DNA-based vaccines co-administered with molecular cytokine adjuvants as part of a vaccine cocktail has been demonstrated to boost the adaptive immune response (5). Recently IL-12 as a vaccine molecular adjuvant has been shown to augment the T cell immunity induced by a DNA vaccine in humans (4). IL-12 was particularly effective in expanding CD4 and CD8 immunity but less effective, in driving strong B cell immunity. Building on this important recent success is an area of great importance. We therefore employed a DNA vaccination approach to investigate the inclusion of Interleukin 33 (IL-33) to further enhance, both arms of the adaptive immune responses.

IL-33 is a member of the IL-1 family of cytokines that is constitutively expressed in the nucleus of epithelial and endothelial cells (6,7). IL-33 is classified as an alarmin-like molecule, whose release during cell injury signals tissue damage to local immune cells (7). Alarmin IL-33 has been shown to have pleiotropic cytokine activities such as mediating diverse pro-inflammatory responses (9,10), activation and recruitment of

antigen-presenting cells (11), enhancing adaptive immunity (12,13), and wound healing (14). To date, IL-33 has been studied primarily in the context of T helper type 2 (T_H2) immune responses associated with modulating inflammatory disorders such as asthma and atopic dermatitis (6,15,16-18). More recently though, IL-33 has been reported to activate CD8 T cells and influence the development of protective anti-viral CD8 T cells against infections in mice (12). However, the role of IL-33 in the induction of vaccine-induced, antigen-specific T_H1 and CD8 T cell immunity remains to be determined. Two different biologically active forms of IL-33 exist: full-length IL-33 (proIL-33) and mature IL-33 (mtrIL-33) (18). ProIL-33 is thought to be the most biologically active form promoting inflammation, while the function of mtrIL-33 in modulating immune responses remains more elusive (7,15,18). Therefore, we investigated whether the two isoforms of IL-33 (proIL-33 and mtrIL-33) can function as vaccine adjuvants to augment adaptive immune responses (both T_H1 and CD8 T cell responses) and induce anti-tumor immunity using a murine model for HPV-associated cancer.

In this study, we demonstrate that IL-33 can act as a potent cell-mediated adjuvant using the DNA vaccine platform. Its adjuvant activity skews towards the T_H1 axis, and not to the T_H2 axis. We show that IL-33 can be effective as an adjuvant in either form – its uncleaved “pro” form or its “mature” state, a shorter form that results from cleavage by cellular enzymes (10,17,19). Both IL-33 isoforms when combined with an HPV16 E6/E7-encoded DNA vaccine enhance the adaptive effector and memory immune responses, but pro IL-33 was more potent at also expanding the humoral immune response. We show that both immunoadjuvant IL-33 isoforms induce potent anti-tumor immunity and regression of established TC-1 tumor-bearing mice. Using the P14 LCMV D^bGP33 transgenic mouse model, we show that immunoadjuvant IL-33 can significantly expand the magnitude of Ag-specific CD8 T cell responses and elicit potent

effector-memory CD8 T cells. Our findings reveal that IL-33 can be an effective adjuvant to drive CD4 immunity, humoral immunity and to generate effective CD8 mediated protective immunity against cancer and potentially have application in treatment of chronic viral infections.

METHODS

Plasmid Construction

The GenBank sequence NM_001164724.1 (accession no: Q8BVZ5.1) for mouse IL-33 was used to synthesize full-length (proIL-33) and mature IL-33 (mtrIL-33) (aa 109-266) plasmid DNA constructs. Each construct had highly efficient immunoglobulin E (IgE) leader sequence inserted at the 5'end of the gene. The constructs were commercially synthesized and optimized as described previously (20). Plasmid expressing HPV 16 ConE6E7 was prepared as previously described (21). The GP33 construct was provided by Dr. Rafi Ahmed of Emory University, Atlanta GA, USA and used as described (22).

Transfection and Expression of Plasmids

ProlL-33 and mtrIL-33 construct expression was confirmed by Western Blot and Immunofluorescence microscopy in Human Rhabdomyosarcoma (RD) cells. RD cells were cultured in 6-well plates and transfected with the constructs (pVAX as control) using LipofectamineTM2000 (Invitrogen) following the manufacturer's protocol. Forty-eight hours later cells were lysed using modified RIPA cell lysis buffer and cell lysate was collected. Western blot analysis was performed with an anti-IL33 monoclonal antibody (R&D systems) and visualized with horseradish peroxidase (HRP)-conjugated anti-rat IgG (Cell Signaling) using an ECL western blot analysis systems (GE Amersham). In addition, supernatants were also collected at 48 hours after transfection and cytokine secretion was examined by mouse/rat IL-33 Quantikine ELISA kit (R&D Systems) according to manufacturer's protocol. An indirect immunofluorescence microscopy assay was also utilized to confirm expression of both IL-33 isoforms. Briefly, RD cells were plated on two-well chamber slides (BD Biosciences) and grown to 70%

confluence overnight in a 37 incubator with 5% CO₂. The cells were transfected with 1ug of IL-33 constructs and the control plasmid pVAX (1 ug/well) using TurboFectin™8.0 Transfection Reagent (OriGene) according to the manufacturer's instructions. Forty-eight hours later the cells were fixed on slides using ice cold methanol for 10 min. The cells were stained with anti-IL-33 mouse monoclonal antibody (R&D Systems, Minneapolis, MN) and subsequently incubated with Alexa 555-conjugated anti-rat secondary antibody (Cell Signaling). Slides were mounted using Fluoromount G with Dapi (Southern Biotechnology). Images were analyzed by florescence microscopy (Leica DM4000B, Leica Microsystems Inc, USA) and quantification was conducted using SPOT Advanced software program (SPOT™ Diagnostic Instruments, Inc).

Animals

Female 8-week-old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The P14 mice bearing the D^bGP33-specific T-cell receptor were a kind gift from Dr. John Wherry of the University of Pennsylvania. To generate the "P14 chimera" mice, 1.6×10^5 naïve T-cell receptor transgenic T cells were adoptively transferred into naïve B6 mice. All animals were conducted and maintained in accordance with the National Institutes of Health and the University of Pennsylvania IACUC guidelines.

Immunization/EP of mice

Mice were immunized three times at three-week intervals in the tibialis anterior muscle. *In vivo* Electroporation (EP) was delivered, with the CELLECTRA adaptive constant current EP device (Inovio Pharmaceuticals, Blue Bell, PA), at the same site immediately

following vaccination as described (20). The mice (n=4-5) were immunized with either 5µg pVAX1 or 5µg ConE6E7 alone or with various amounts of proIL-33 and mtrIL-33 constructs, depending on the experiment. The GP33 construct was administered at 5µg. All studies were repeated twice.

ELISpot assays

Spleens were harvested 8 days following the final immunization as previously described (20). After spleens were harvested and processed both IFN γ and IL-4 ELISpot assays were performed to determine antigen-specific cytokine secretion from immunized mice as described previously (20,21,22). A set of peptides (15 amino acid residues overlapping by 8 amino acids) representing the entire consensus E6/E7 fusion protein sequence of HPV16 was synthesized from GenScript. This set of peptides was combined into two pools, spanning the length of the E6 and E7 antigens as previously described (21). Concavalin A (Sigma-Aldrich, St. Louis, MO) at 5µg/ml was used as positive control and complete culture medium was used as negative control. Spots were enumerated using an automated ELISPOT reader (Cellular Technology, Shaker Heights, OH).

Flow Cytometry

Lymphocytes were isolated and processed from the spleen and peripheral blood as previously described (20, 23, 24). Splenocytes were added to a 96-well plate (1×10^6 /well) and were stimulated with pooled HPV-16 E6/E7 pooled peptide for 5-6 hours at 37C/5% CO₂ in the presence of Protein Transport Inhibitor Cocktail (Brefeldin A and Monensin) (eBioscience) according to the manufacturer's instructions. The Cell

Stimulation Cocktail (plus protein transport inhibitors) (phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin) (eBioscience) was used as a positive control and R10 media as negative control. In cultures being used to measure degranulation, anti-CD107a (FITC; clone 1D4B; Biolegend) was added. All cells were then stained for surface and intracellular proteins as described previously (20). Briefly, the cells were washed in FACS buffer (PBS containing 0.1% sodium azide and 1% FCS) before surface staining with flouorochrome-conjugated antibodies. Cells were washed with FACS buffer, fixed and permeabilized using the BD Cytofix/Ctyoperm TM (BD, San Diego, CA, USA) according to the manufacturer's protocol followed by intracellular staining. The following antibodies were used for surface staining: LIVE/DEAD Fixable Violet Dead Cell stain kit (Invitrogen), CD19 (V450; clone 1D3; BD Biosciences) CD4 (FITC; clone RM4-5; Ebioscience), CD8 (APC-Cy7; clone 53-6.7; BD Biosciences), CD44 (A700; clone IM7; Biolegend); KLRG1 (FITC; clone 2F1; eBioscience). Major histocompatibility complex class I peptide tetramer to LCMV-GP33 was used as described previously (4,5). For intracellular staining the following antibodies were used: IFN γ (APC; clone XMG1.2; Biolegend), TNF α (PE; clone MP6-XT22; ebioscience), CD3 (PerCP/Cy5.5; clone 145-2C11; Biolegend). All data was collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR) and SPICE v5.2 (free available from <http://exon.niaid.nih.gov/spice/>). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals. Dead cells were removed by gating on a LIVE/DEAD fixable violet dead cell stain kit (Invitrogen) versus forward scatter (FSC-A)

Antigen-specific Antibody Determination

The measurement of IgG antibodies specific for viral genes E6 and E7 was performed by ELISA (enzyme linked immunosorbent assay) in both immunized and controlled mice. The plates were coated with 1ug/ml of each protein (ProteinX Lab) and incubated overnight at 4 degrees. After washing, plates were blocked with 10% fetal bovine serum (FBS) in 1x phosphate-buffered saline (PBS) for 1 hour at room temperature. Plates were then washed again and serum was added at a 1:25 dilution in 1% FBS + PBS + 0.05% Tween-20 and incubated at room temperature for 1 hour. After another wash, goat anti-mouse IgG HRP (Santa Cruz) at a 1:5000 dilution was added to each well and incubated for 1 hour at room temperature. Following a final wash, the reaction was developed with the substrate 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) and stopped with 100ul of 2N sulfuric acid/well. Plates were read at 450nm on Glomax Multi-Detection System (Promega). All serum samples were tested in duplicate. The amount of antigen specific IgE was also determined using a similar ELISA protocol using the secondary rat anti-mouse IgE HRP antibody (Southern Biotech). Total IgE was determined using GenWay's mouse IgE kit. The manufacturer's protocol was followed with serum dilutions at 1:50. All serum samples were tested in duplicate.

Tumor Cell line

TC-1 cells were purchased from ATCC and cultured as previously described (26). The TC-1 cell line was a graciously given gift from Dr. Yvonne Paterson of the University of Pennsylvania, Philadelphia, PA, USA. The TC-1 cell line is well characterized, constitutively expresses E6 and E7, and is highly tumorigenic (26, 27). TC-1 cells were prepared and mixed with Matrigel (BD Bioscience) for subcutaneous (s.c.) tumor

implantation.

***In vivo* tumor treatment (regression) study**

Female B6 mice were separated into four groups of 10 mice each and 5×10^4 TC-1 cells were s.c. implanted into the flanks of each wild-type female B6 mice. On days 4, (after tumor implantation and when tumors reached 3mm), each group of mice was immunized i.m./EP with pVAX, ConE6E7, ConE6E7 proIL-33 and ConE6E7 mtrIL-33, respectively and boosted on day 11 and 18. Mice were monitored twice a week for tumor growth and were measured as described previously (21,27).

Statistical analysis

Student's *t-test* was applied for comparison of the quantitative data of the cellular immune response and tumor diameters. In this study, $p < 0.05$ were regarded as statistically significant.

RESULTS

Construction and expression of IL-33 isoforms

Two IL-33 adjuvants constructs (pro-IL33 and mtrIL-33) were designed and generated to test our working hypothesis (**Figure 2.1A**). To determine the expression of both IL-33 isoforms, human rhabdomyosarcoma (RD) cells were transfected separately with each construct, and expression was assessed by Western immunoblotting. A ~20kDA protein was observed for mtrIL-33 and a ~30kDA and ~20kDA protein size was observed for proIL-33, in cell lysates harvested 48 hours after transfection using an anti-IL33 monoclonal antibody (mAb) for detection (**Figure 2.1B**). For a comparative control, no protein band could be detected in the negative pVAX control. To examine the cytokine secretion of both isoforms, cell supernatants were obtained 48 hours after transfection in RD cells and the detection of cytokine secretion into the extracellular environment were carried out by enzyme-linked immunosorbent assays (ELISAs). As shown in **Figure 2.1C**, supernatants from mtrIL-33 and proIL-33 transfected RD cells contained mtrIL-33 and proIL-33 at concentrations of roughly 20,000 pg/ml and 600 pg/ml, respectively. Finally, the expression for both IL-33 isoforms was further confirmed using immunofluorescent staining using an anti-IL33 mAb. ProIL-33 can act both as a secreted cytokine and as a nuclear binding factor (19). ProIL-33 nuclear localization is mediated by the nuclear localization signal in its N-terminus, which also contains a chromatin-binding motif (**Figure 2.1A**). However, the cleavage of proIL-33 into mtrIL-33 yields a truncated IL-33 that lacks the nuclear localization signal found in proIL-33. As projected, high nuclear expression with some cytoplasmic expression was observed in the proIL-33 transfected cells (**Figure 2.1D**, bottom). This is an important observation which supports previous findings showing that proIL-33 cytokine can also be expressed

and found in the cytoplasm (27). In contrast, only high cytoplasmic expression was visualized in the mtrIL-33 transfected cells shown in **Figure 2.1D**, middle.

IL-33 adjuvant isoforms enhance potent HPV-specific cellular immune responses following vaccination

A quantitative ELISpot assay was used to determine the number of antigen-specific IFN γ secreting cells in response to stimulation with the E6 and E7 peptide pool. As we have reported, electroporation (EP) improves the immunogenicity or potency of DNA vaccines by increasing antigen expression (28,29), thus we performed ConE6E7/EP intramuscular (i.m.) vaccination in C57BL/6 (B6) mice (n=5) with a dosage of 5 μ g alone or in combination with either mtrIL-33 or proIL-33 at various doses followed by EP. One week after final immunization we monitored the degree of immune responses by isolating splenocytes for further analysis (**Figure 2.2A**). As shown in **Figure 2.2B**, the critical role of IL-33 to drive T_H1-polarized immune responses is clearly demonstrated. Co-immunization with both adjuvant cytokine-encoding plasmids induced higher numbers of E6- and E7-specific IFN γ secreting T cells at all doses when compared with ConE6E7 alone-vaccinated mice (~500 SFU per million splenocytes). As noted in **Figure 2.2B**, the optimal dose of either the mtrIL-33 or proIL-33 (7 μ g) resulted in a total 4 and 3.5-fold increases in IFN γ responses, respectively. Due to earlier reports suggesting that IL-33 was a key cytokine in the induction and support of a T_H2 response (6,15,16), we assessed whether IL-33 induced the prototypical T_H2 cytokine, Interleukin-4 (IL-4) via IL-4 ELISpot. Our data reveals that neither form of IL-33 drove a robust secretion of IL-4 (**Figure 2.2C**). Instead, IL-33 as an adjuvant skewed towards the T_H1

biased axis, and not the T_H1 cytokine associated immune responses as originally described (18).

IL-33 enhances HPV antigen-specific CD4 and CD8 T cell immunity

We next characterized the antigen (Ag)-specific phenotype and cytokine production profile of memory T cells generated, using the 7 μ g dose that induced the optimal adjuvant affect as shown in **Figure 2.2B**. Given the importance of multifunctional CD4 and CD8 T cell immunity in the elimination of HPV16-infected cells (5, 30-33), we measured the ability of vaccine-induced Ag-specific T cell populations to secrete IFN γ and TNF α , in response to *ex vivo* E6/E7 pooled peptide stimulation in the spleens. Our gating strategy for intracellular cytokine flow-cytometry analysis is depicted in **Figure 2.3A**. Compared with ConE6E7 vaccination alone, the ConE6E7 co-administered with mtrIL-33 and proIL-33 elicited higher frequency of HPV-specific CD4 T cells producing either total IFN γ (mtrIL-33: 0.21%; proIL-33: 0.25%), total TNF α (mtrIL-33: 0.25%; proIL-33: 0.39%) and dual IFN γ /TNF α (mtrIL-33: 0.12%; proIL-33: 0.15%) (**Figure 2.3B-E**). In terms of CD8 T cells, we observed that vaccination with both IL-33 isoforms elicited substantially higher frequencies of HPV-specific CD8 T cells producing total IFN γ (mtrIL-33: 3.68%; proIL-33: 3.50%), total TNF α (mtrIL-33: 3.11%; proIL-33: 3.13%) and dual IFN γ /TNF α (mtrIL-33: 2.83%; proIL-33: 2.75%) (**Figure 2.4A-C**). The same trend was seen with the frequency of Ag-specific CD8⁺ T cells secreting IFN γ alone and TNF α alone (**Figure 2.4D**). Overall, both immunoadjuvant IL-33 isoforms produced similar amounts of Ag-specific CD4 and CD8 T cell responses, with cytokine production mediated mainly by CD8 T cells. The high frequencies of effector cells secreting anti-viral cytokines are indicative of the adjuvant effects of IL-33 to enhance vaccine potency.

Given the importance of cytotoxic CD8 T lymphocytes (CTLs) functionality as critical components in protection (34), we characterized the cytotoxic potential of vaccine induced CD8 T cells undergoing degranulation. CD8 T cells isolated from mice vaccinated with adjuvant showed a higher frequency of the degranulation marker, CD107a (mtrlL-33: 4.4%; proIL-33: 4.9%), compared to mice that received the ConE6E7 constructs alone (**Figure 2.4E**). More interestingly, the HPV-adjuvanted vaccines elicited substantially higher frequencies of plurifunctional effector CD8 T cells co-expressing CD107a/IFN γ /TNF α (mtrlL-33: 2.5%; proIL-33: 2.5%), compared to the ConE6E7 construct alone (**Figure 2.4F**). These results indicate the adjuvant potential of IL-33 to induce functional effector cytotoxic CTLs, which have a phenotype suggesting the cells ability to clear HPV16 infected cells.

IL-33 role in the induction of humoral responses

Identifying potent adjuvants that not only mediate protective cell-mediated immune responses, but can also induce humoral immune responses, will be ideal for enhancing effective prophylactic and therapeutic vaccines against a variety of microbial infections. Thus, to determine whether mtrlL-33 and proIL-33 influence the level of circulating HPV E6- & E7-specific antibodies, we analyzed humoral responses by ELISA using collected sera obtained one-week post final vaccination. As shown in **Figure 2.5A**, only co-immunization with proIL-33 significantly induced E7-specific total IgG compared to other immunized groups. No E6-specific antibodies were induced or detected (data not shown). In addition, because reports have indicated that IL-33 plays a role in allergic responses we examined E7-specific IgE and total IgE responses in the sera. As illustrated in **Figure 2.5B** and **2.5C**, the adjuvant effects of mtrlL-33 and proIL-33 did not

drive enhanced levels of IgE responses compared to control vaccinated groups. This is consistent with the low induction of IL-4 responses shown in **Figure 2.2C**, as IL-4 is known to drive IgE class-switch (35). These results supported that IL-33 adjuvant effects in a DNA vaccination setting do not induce T_H2-associated responses. Interesting, only the combination of HPV and proIL-33 increased Ag-specific IgG humoral responses, indicating its role as an effective adjuvant to enhance both Ag-specific cell-mediated and humoral responses.

IL-33 immunoadjuvants induce potent anti-tumor immunity and regression of established TC-1 tumor-bearing mice

A strong frequency of anti-HPV CD4 T_H1 and CD8 T cell immunity has been considered a critical characteristic of an effective therapeutic T cell-based vaccine designed to control and eliminate established pre-existing HPV infections and associated lesions (32,36). Given the results that IL-33 acts as a cell-mediated adjuvant eliciting potent HPV Ag-specific T_H1-and CD8-biased T cell immune responses, we performed an *in vivo* tumor therapy study to determine the therapeutic efficacy of IL-33 immunoadjuvants in TC-1 tumor bearing mice. HPV16 E6/E7-expressing TC-1 tumors (5x10⁴) cells were implanted in naïve B6 recipient mice. Four days after TC-1 cell implantation, tumors were measured (tumors had reached an average size of 3 mm) and groups of mice (n=10) were immunized with pVAX, ConE6E7 (5µg) alone, or ConE6E7 co-administered with 7µg of mtrIL-33 or proIL-33, followed with two boosts at one week intervals as outlined in **Figure 2.6A**. As shown in **Figure 2.6B**, tumor growth was substantially rejected in the mtrIL-33 and proIL-33-adjuvanted groups compared with controls. The IL-33 groups remained tumor free until day 42, with the exception of one

mouse in the mtrIL-33-adjuvant group. Meanwhile, only 6 mice in the ConE6E7-vaccinated group were tumor free after 42 days and in the control group all mice had died by day 28. Furthermore, as shown in **Figure 2.7**, both IL-33 isoforms can maintain and elicit anti-tumor memory responses similar to ConE6E7. Clearly, ConE6E7 can easily prevent E6/E7 tumor growth, but have difficulties curing in a tumor therapy study, however the inclusion of IL-33 makes a substantial difference (**Figure 2.6B**). Thus, our data illustrates that HPV-specific T cell immunity induced by both immunoadjuvants provides substantial protective anti-tumor immunity by further delaying or rapidly inducing complete regression of established TC-1 tumors.

IL-33 adjuvant expands Ag-specific CD8 T cell responses and elicits potent CD62L⁺KLRG1⁺ effector-memory CD8 T cell responses after vaccination

Given the increase of Ag-specific CD8 T cell responses and the remarkable display of complete tumor regression elicited by immunoadjuvant proIL-33, we examined whether the 100% protective efficacy of proIL-33 was due to its ability to rapidly expand the effector memory CD8 Ag-specific T cell responses. To achieve this goal, we took advantage of the P14 (D^bGP33-specific T cell receptor (TCR)) mouse model, which is a great model for tracking populations of T cell subsets. Therefore, to investigate CD8 T cell expansion during vaccination with proIL-33, we transferred ~150,000 Ly5.1⁺ naïve P14 TCR transgenic CD8 T cells into (n=4/group) naïve wild type recipients to make “P14 chimeric mice” that were subsequently vaccinated with GP33 alone and GP33 coimmunized with proIL-33. The frequency of the Ag-specific CD8⁺ T cells responses was monitored in the blood during the course of a prime and boost DNA vaccination with or without proIL-33 adjuvant (**Figure 2.8**). As shown in **Figure 2.8A** and **Figure 2.8B**,

the GP33-proIL-33 adjuvanted group dramatically increased the frequency of P14 CD8⁺Ly5.1⁺ T cells in the blood, compared to the non-adjuvanted group. This significantly increased frequency (~5-fold) of Ly5.1⁺ CD8⁺ T cells peaked at ~14 dpv (days post vaccination) compared to the GP33 immunized group which reached its peak at ~21 dpv. Furthermore, seven days after homologous boosting (48 days after initial vaccination), proIL-33 immunoadjuvant markedly increased the frequency of Ag-specific CD8⁺ T cells compared to control group (**Figure 2.8B**).

Several studies have suggested that effector CD8 T cells are the optimal subset for protective immunity and pathogen control (37-39). It has been proposed that a predominant CD62L⁻KLRG1⁺ effector-memory T cell response may be a vital prognostic for the efficacy of therapeutic cancer vaccines (40). Thus, starting at 14 dpv we examined the phenotype of the effector CD8 T cells within the vaccine-induced P14-specific CD8 T cell population based on the cell surface expression markers: Ly5.1, CD62L and KLRG1 (**Figure 2.8C**). As shown in **Figure 2.8C**, the percentages of CD62L⁻KLRG1⁺ effector memory cells were significantly higher in the proIL-33 adjuvant group compared to the GP33-only vaccinated group. Secondary memory cells showed a greatly expanded population of KLRG1⁺ T cells in both groups after homologous DNA boosting, 48 days after initial immunization. The effector-memory responses remained significantly higher in the proIL-33-adjuvanted group compared to GP33-alone group. Together, these results support the notion that IL-33 increases the formation of Ag-specific CD8 T cells and that IL-33 can enhance clonal expansion of the effector memory pool (12).

FIGURES

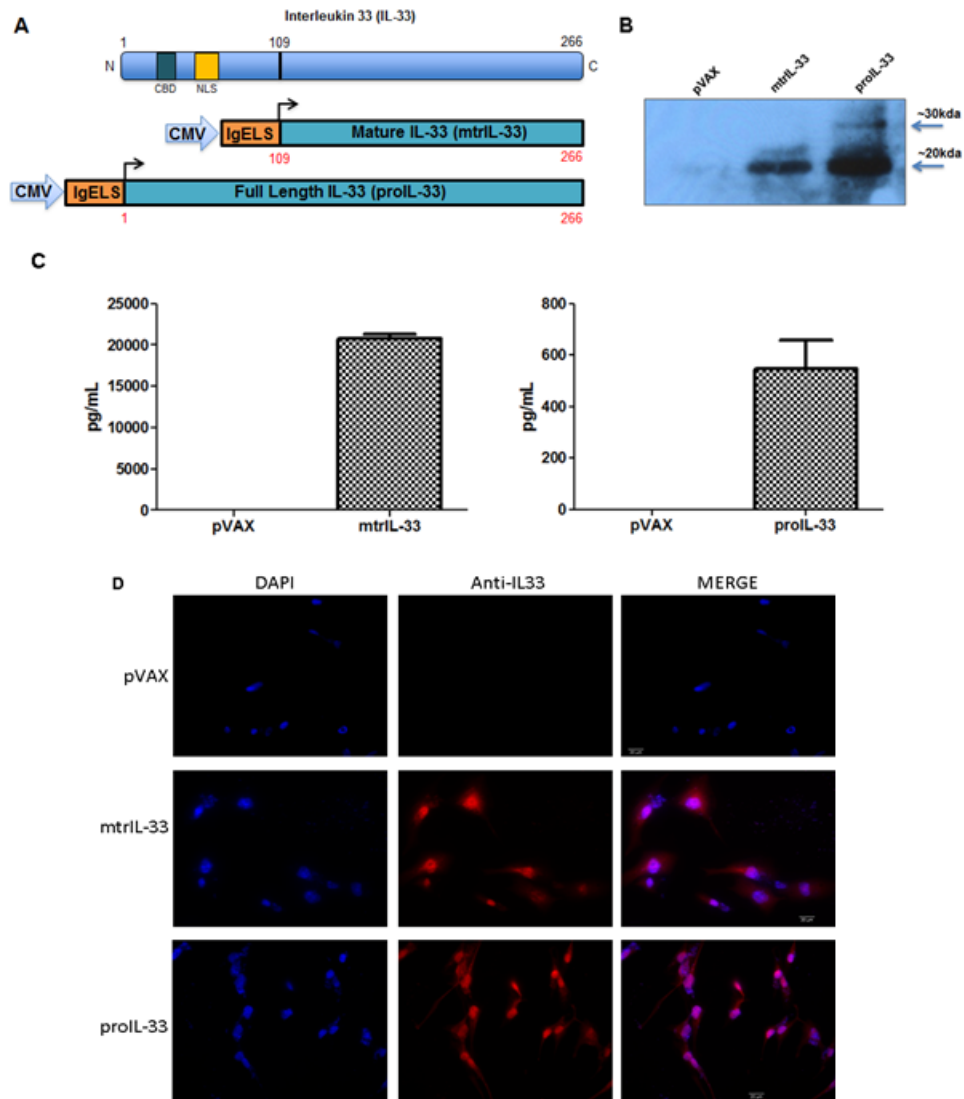


Figure 2.1. Expression and secretion of mtrIL-33 and proIL-33 DNA vaccine constructs. (A) Schematic representation of IL-33 protein and the IL-33 adjuvant constructs encompassing the proIL-33 and mtrIL-33 under the CMV promoter. All constructs contain an IgE leader sequence. The N-terminus domain of IL-33 contains a chromatin-binding motif (CBM) and nuclear localization signal (NLS). (B) Expression of mtrIL-33 and proIL-33 constructs in RD cells as examined by Western blot analysis. Labeled lanes show proteins detected by anti-IL33 mAb. Smaller band represents mtrIL-33, while the larger band represents proIL-33. (C) Secretion of IL-33 from transfected RD cells was confirmed via ELISA. Data shows the means with standard error of the means (SEM) for two replicate assays. (D) Detection of expression of mtrIL-33 and proIL-33 via Immunofluorescence microscopy

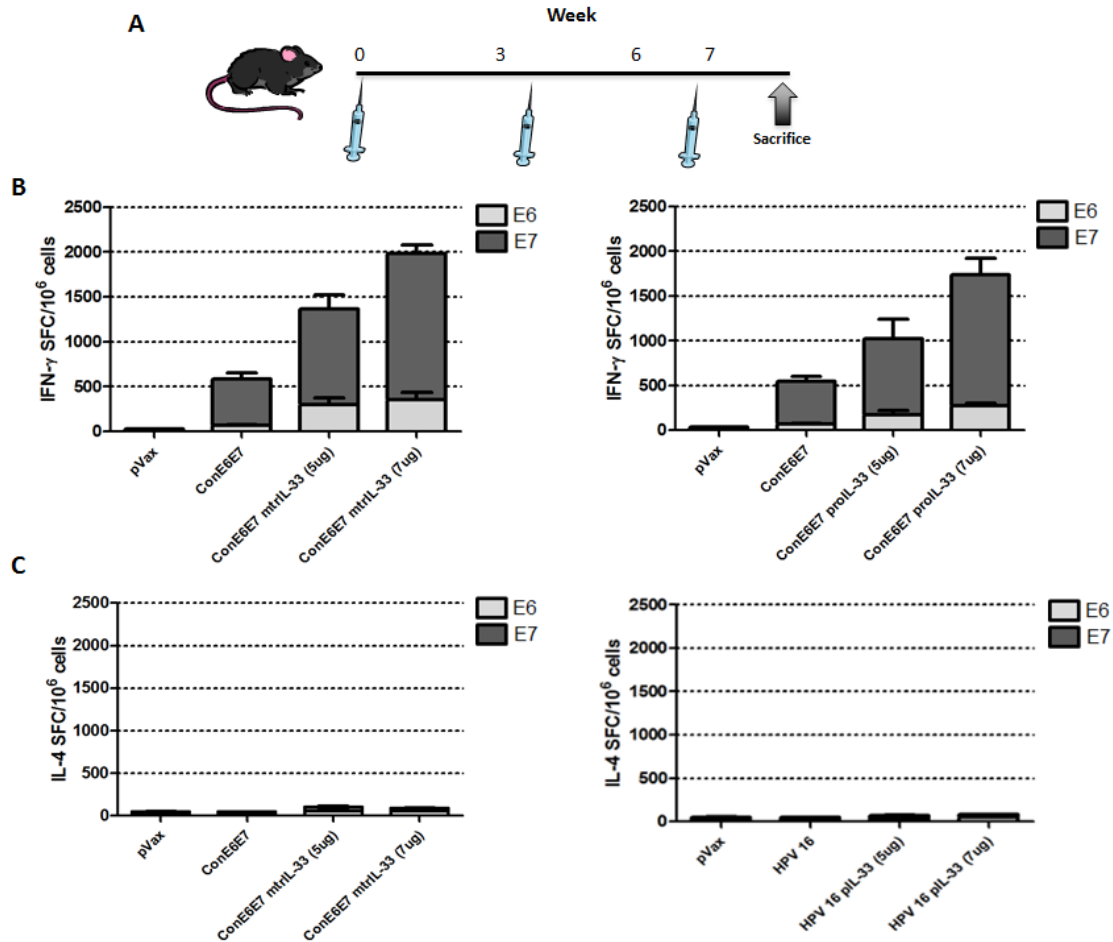


Figure 2.2. Immunoadjuvants IL-33 isoforms enhance strong HPV16 E6- and E7-specific IFN- γ immune responses, but no IL-4 responses. (A) DNA vaccine immunization schedule for adjuvant study. C57BL/6 (B6) mice (n=5 per group) were immunized at weeks 0, 3, and 6 with HPV16 consensus E6/E7 (ConE6E7) construct with or without adjuvant via intramuscular/EP and spleens were harvested one week post final immunization to assess the cellular immune responses. (B) The induction of a Th1 response is shown by the frequency of HPV16 E6 and E7-specific IFN γ spot-forming units (SFU) per million splenocytes determined by IFN γ ELISpot assay. (C) Antigen-specific IL-4 responses measured by IL-4 ELISpot assay. Experiments were performed independently at least two times with similar results.

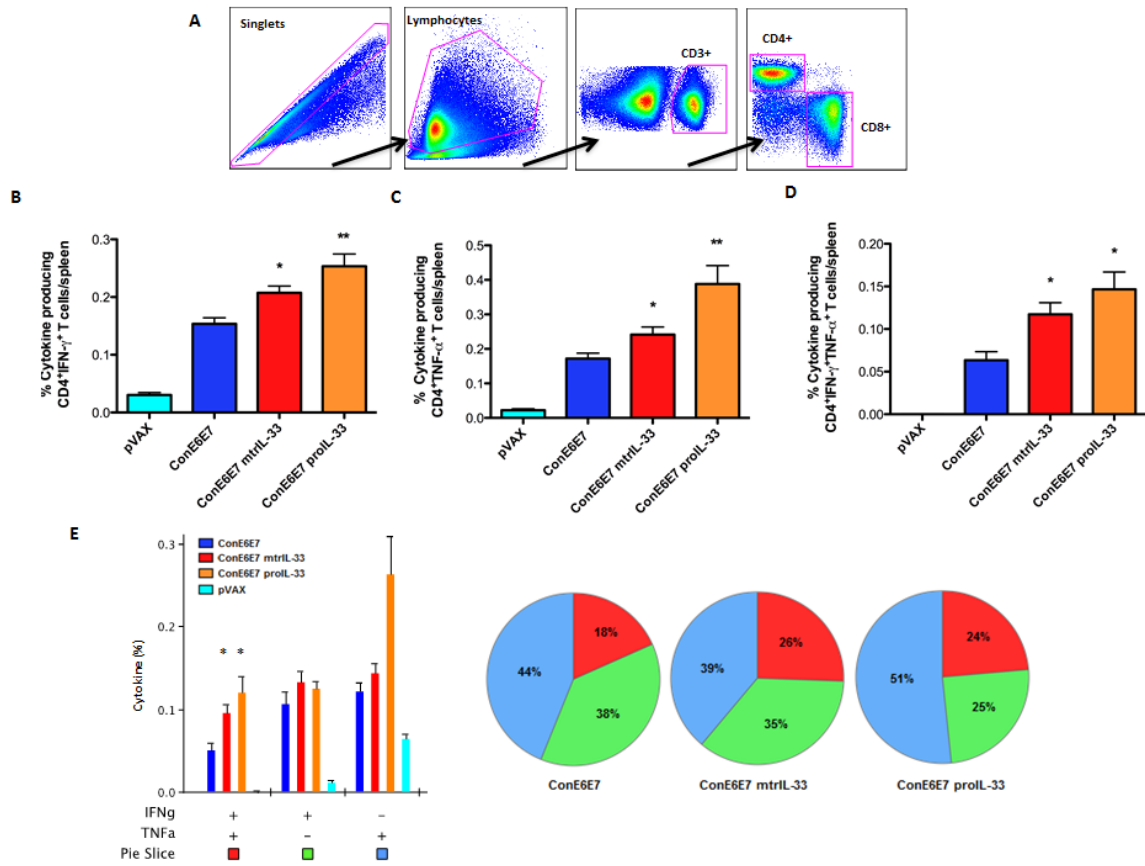


Figure 2.3. Cytokine frequencies of specific CD4 T cells induced by immunoadjuvants mtrIL-33 and proIL-33. (A) Depicted is the gating strategy used for identifying Ag-specific T cell populations. (B) Column graphs depicting E6/E7-specific CD4⁺ T cells releasing the cytokines IFN γ (C) TNF α and (D) double-positive producing cells (and pVAX control). (E) Column graph shows plurifunctional subpopulations of single- and double-positive CD4 T cells releasing the cytokine IFN γ and TNF α . Pie charts show the relative proportion of each cytokine subpopulation to Ag-specific stimulation. Experiments were performed independently at least two times with similar results with five mice per group.

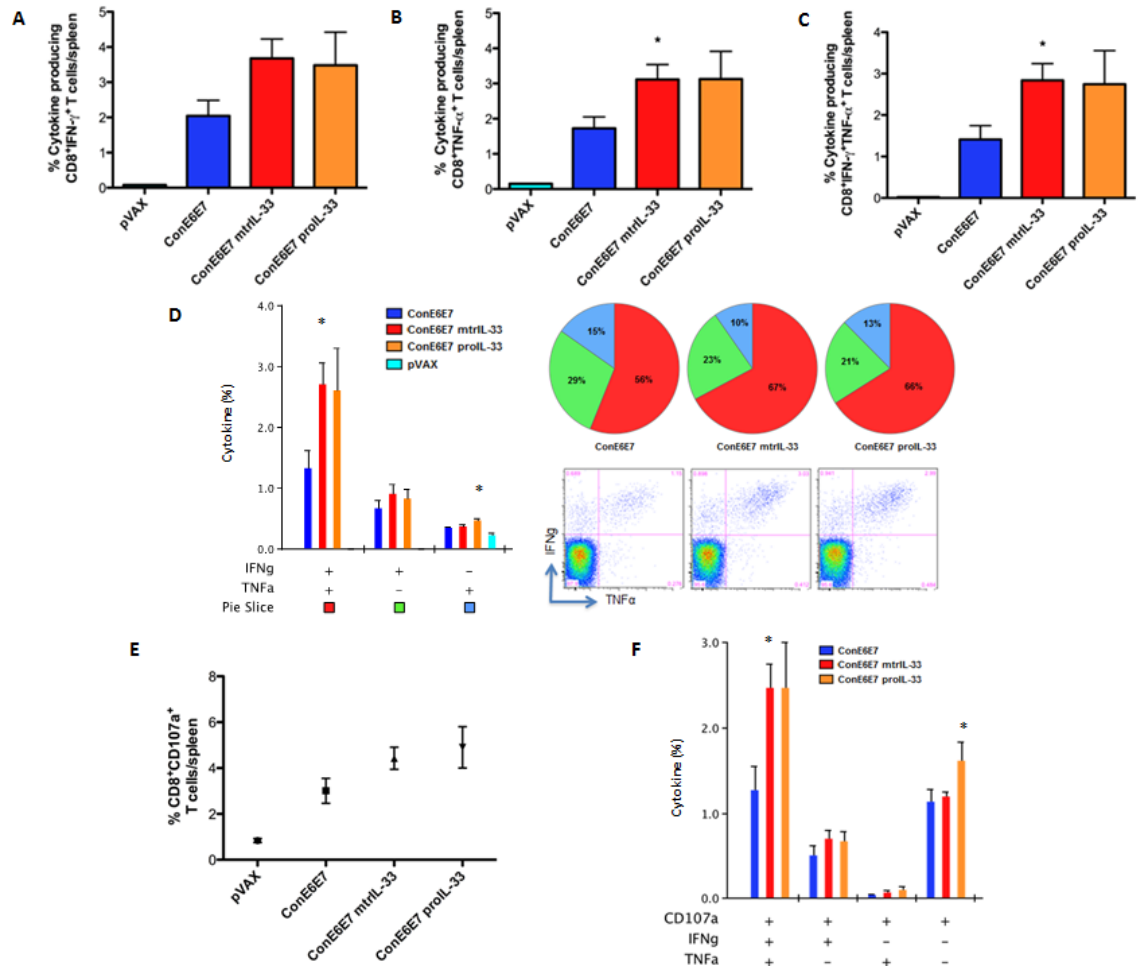


Figure 2.4. Cytokine frequencies of specific CD8 T cells induced by immunoadjuvants mtrlL-33 and proIL-33. (A) Column graphs depicting E6/E7-specific CD8⁺ T cells releasing the cytokines IFN γ (B) TNF α and (C) double-positive producing cells (and pVAX control). (D) Column graph shows plurifunctional subpopulations of single- and double-positive CD8 T cells releasing the cytokine IFN γ and TNF α . Pie charts show the relative proportion of each cytokine subpopulation to Ag-specific stimulation. Dot plots, representative of four mice is also shown in (D), depicting double positive cytokine expressing CD8 cells after stimulation with pooled E6/E7 peptide. (E) Antigen-specific cytolytic degranulation T cells were measured by degranulation marker expression, CD107a. (F) Cytokine profile of the cytolytic phenotype. Experiments were performed independently at least two times with similar results with five mice per group.

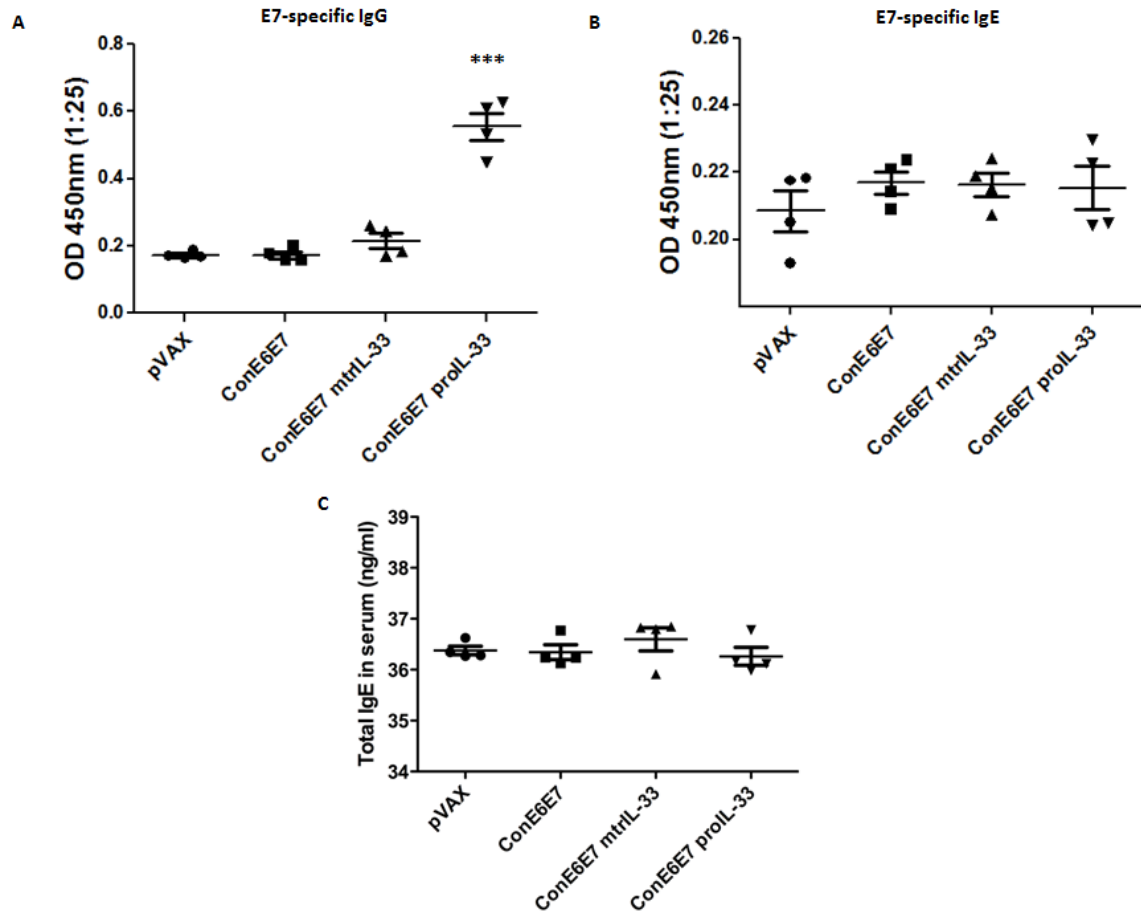


Figure 2.5. Humoral responses of ConE6E7 with and without mtrlL-33 and proIL-33 adjuvants. Blood collected from control (pVAX) and immunized mice (n=4) was analyzed for humoral responses via ELISA one week after last immunization. (A) Specific total IgG antibodies against HPV16 E7 (B) Specific IgE antibodies against HPV16 E7. (C) Total IgE antibodies detected in the serum. Experiments were repeated two times with similar results.

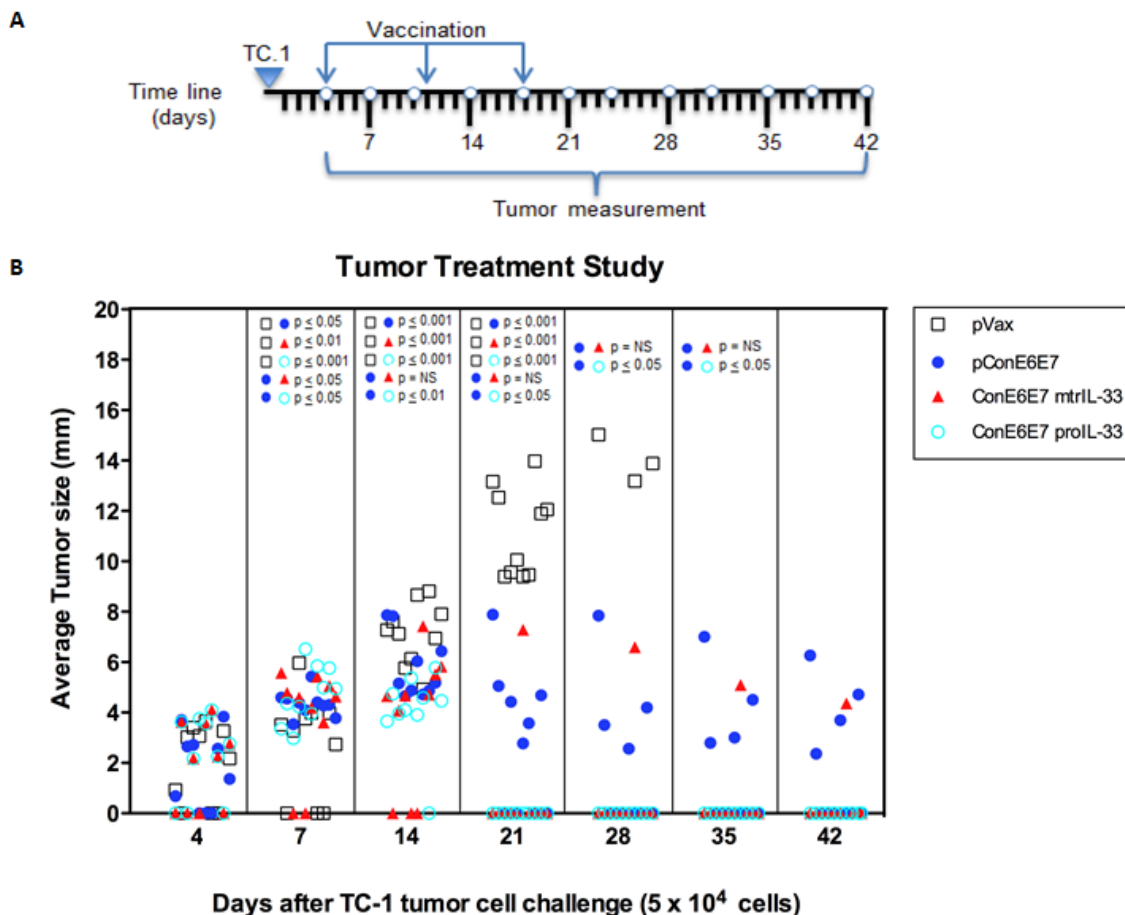


Figure 2.6. Vaccination with IL-33 adjuvants induces regression of established TC-1 tumors. (A) Schematic illustration of the time line of therapeutic study regimen. (B) Groups of B6 mice (10 mice/group) were s.c. challenged with 5×10^4 TC-1 tumor cells. Tumors were measured twice a week in two dimensions with electronic calipers and data are presented with the average of these values over time for each individual mouse. Mice were sacrificed when tumor diameter reached approximately 2.0 cm. Tumor measurements for each time point are shown only for surviving mice. pVAX immunized mice served as negative control.

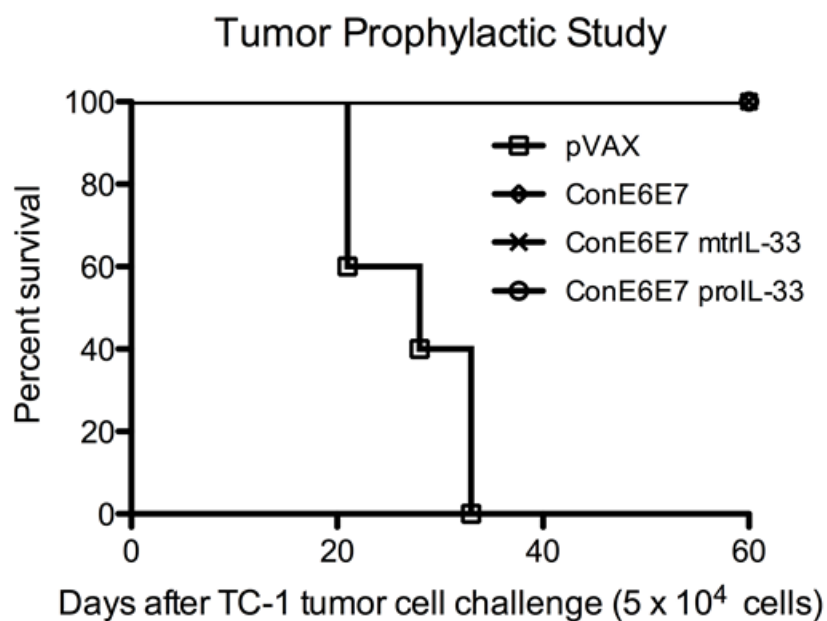


Figure 2.7. Protective efficacy maintained by mtrIL-33- and proIL-33-adjuvanted ConE6E7. In a prophylactic study, groups of B6 (10 mice/group) were immunized (three times at three week intervals) with ConE6E7 with or without $7\mu\text{g}$ of mtrIL-33 or proIL-33 and challenged with 5×10^4 TC-1 cells one week after last immunization to assess the anti-tumor effects. All vaccination groups prevented tumor growth upon TC-1 implantation.

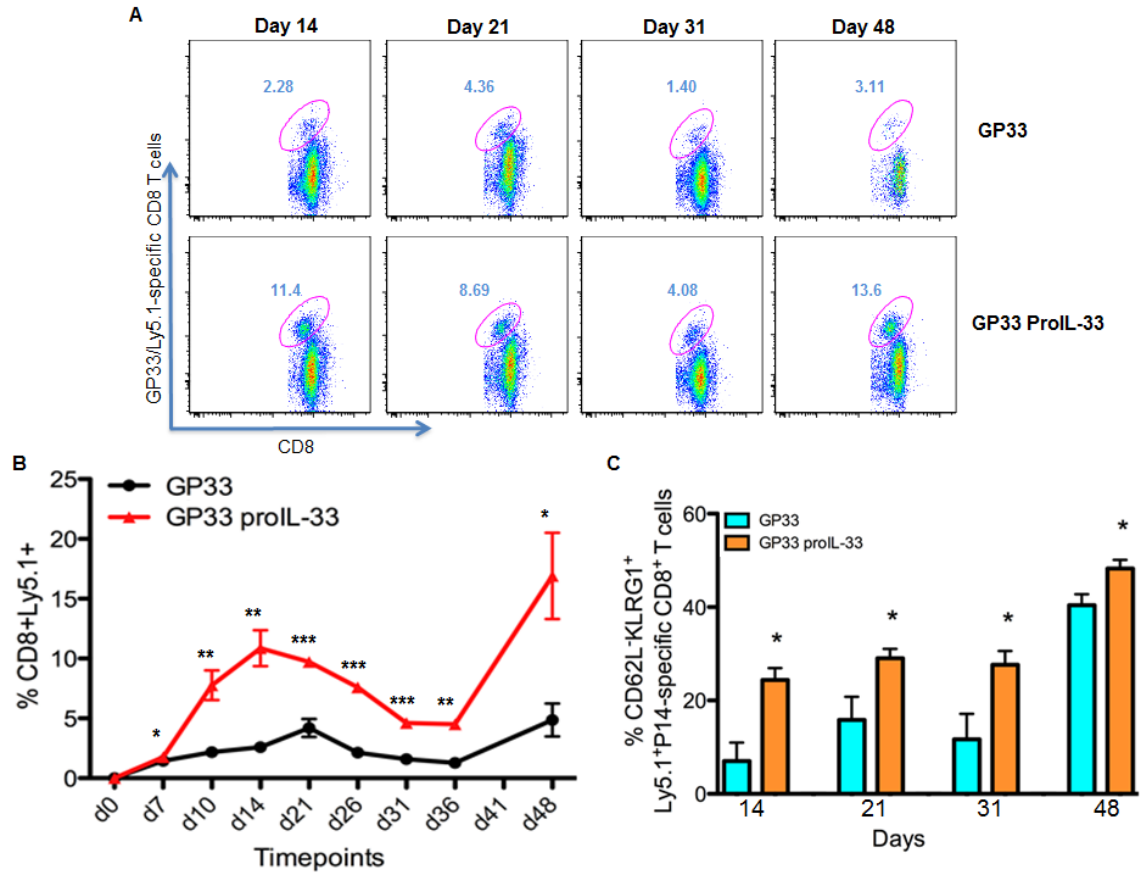


Figure 2.8. Immunoadjuvant IL-33 expands the frequency and effector memory phenotype of the Ag-specific CD8 T cells. P14 chimera mice (n=4) were vaccinated twice (on day 0 and day 41) containing GP33 with or without proIL-33. (A) Displayed are representative fluorescent intensity plots of GP33/Ly5.1-specific CD8 T cells responses in the blood of vaccinated mice at days 14, 21 and 31 after first vaccination and day 48 (day 7 after second immunization). Numbers indicate the percentage of Ag-specific CD8⁺ T Cells within the total CD8⁺ T cell populations. (B) Kinetics of Ly5.1⁺ expression on P14-specific CD8 T cells in PBMC following DNA vaccination with a prime at day 0 and a boost at day 41. (C) Distribution of effector memory CD8⁺ T cell from immunized mice at day 14, 21, 31 after first vaccination and day 48 (day 7 after second vaccination). Data are representative of two independent experiments with four mice per group.

DISCUSSION

In this study, we provide insight on the T_H1-and CD8-biased adjuvant activity of two isoforms of IL-33-encoding plasmids in a DNA vaccine setting. We demonstrate that IL-33 elicits bonafide Ag-specific T_H1cell-mediated immune responses to a consensus HPV16 E6/E7 antigen, but neither IL-4, nor any elevation of IgE levels as previously described. Clearly both isoforms elicited strong HPV16 Ag-specific polyfunctional CD4 and CD8⁺ T cells secreting both anti-viral IFN γ and/or TNF α cytokines, and also induced an increase in the Ag-specific cytolytic effector CD8 T cells undergoing plurifunctional degranulation. More importantly, both IL-33 isoforms were shown to be strong adjuvants when used in conjunction with a therapeutic HPV DNA vaccine to generate robust anti-tumor immunity, facilitating successful tumor regression in established TC-1 tumor-bearing mice.

The major significant difference between proIL-33 and mtrIL-33 was that proIL-33 was able to increase E7-antigen specific IgG levels. However, because mtrIL-33 induced 90% tumor regression, it suggested that T cells mediated the anti-tumor protection, not B-cell responses. Full length IL-33's dual function property, to act not only as a cytokine, but also as a nuclear transcription factor, may explain the increase in antibody responses by proIL-33. Its nuclear localization may have additive effects on modulating the humoral immune responses. However, the specific transcriptional targets of nuclear IL-33 are still unclear. We are currently pursuing understanding its precise role in the nucleus and its association with modulation of immunogenicity. Although the importance of this finding is not yet clear, the data suggests that proIL-33 could also be useful in vaccine strategies aiming to achieve enhanced antibody responses and cellular immunity. This is an area of further investigation.

The specific roles in the protective responses against HPV infection and associated cancers have been attributed to CD8 T cell immune responses, and therefore, are the focus for achieving effective immunity by therapeutic treatments against tumors (5,21, 28, 32). Our results reveal that the cytokine secreting T cell responses induced by IL-33 were mainly mediated by eliciting a high frequency of Ag-specific CD8⁺ T cells co-expressing CD107a/IFN γ /TNF α . While Bonilla *et al.* similarly demonstrated that IL-33 can drive plurifunctional CD8 T cell responses in a viral infection model (12), we further demonstrated that the delivery of IL-33 as an immunoadjuvant can indeed enhance plurifunctional CD8 T cell responses, further expanding the pool of information we now know about IL-33. Consistent with this enhanced polyfunctional anti-HPV effector CD8 and CD4 T cell immunity, mice vaccinated with IL-33 demonstrated remarkable ability to induce anti-tumor immunity and tumor regression in established TC-1 tumor bearing mice (**Figure 2.6B**). The significantly improved vaccine efficacy offered by IL-33 suggests its potential utility as a vaccine adjuvant. Recently, Luzina *et al.* demonstrated that mtrIL-33 induced T_H2 responses *in vivo* via a mouse model of pulmonary infection (18). In contrast, we show *in vivo* that not only proIL-33, but also mtrIL-33, a cleaved form of proIL-33 has pleiotropic properties, and can modulate the immune responses towards a T_H1 and CD8 T cell response. It seems that IL-33 may not be a classical T_H2 cytokine as originally suggested, but under certain conditions can promote T_H1 and CD8 type immunity. It is likely that other immune cells may have accounted for the observed enhancement in T_H1 immunity and tumor regression. For instance, IL-33 has been shown to activate Natural Killer (NK) cells (6,41). However, it is unlikely that NK cells could have accounted for the observed enhancement in CD8 T cell immunity or tumor regression. The HPV E6-E7 vaccine encodes a nuclear antigen that is

not lipid based and not targetable by Fc-Receptor bound antibodies directing NK immunity and can only be a target of CD8 T cells. Furthermore, much prior work in the TC-1 tumor challenge model, including work conducted by our lab, has established that this model is CD8⁺ T cell dependent for protection (26,32,42-44). Nevertheless, further studies will be needed to elucidate under what conditions IL-33 promotes T_H1 and CD8 T cell immunity, and the IL-33 regulatory networks connecting the innate with the adaptive immune response.

It is known that IL-33 exhibits pleiotropic properties and could promote responses other than T_H1, such as activating CD8 T cells (12,13,27). Thus, to investigate the ability of IL-33 to modulate the CD8 T cells we used the P14 mouse model to monitor the expansion of LCMV D^bGP33/Ly5.1⁺ cells in the P14 chimeras after immunizing mice with a cognate viral Ag. We show *in vivo* that IL-33 can modulate the expansion of CD8 T cells in a vaccine setting and observed that inclusion of the IL-33 adjuvant significantly expanded the magnitude of Gp33/Ly5.1⁺-specific CD8 T cell responses in the blood (**Figure 2.8**). These data demonstrate the overall superiority of immunoadjuvant IL-33 in enhancing the Ag-specific CD8 T cells in a DNA vaccine. In addition to implying that IL-33 plays an important role in the expansion of CD8 T cells, it also suggests that IL-33 mediated antitumor immunity and tumor regression in the TC-1 tumor therapy study was probably CD8 T cell related (**Figure 2.6**). Moreover, as shown in **Figure 2.8**, the peak of CD8 expansion (14 dpv) seemed to correlate with the complete tumor regression mediated by proIL-33, which was 17 days post first vaccination (**Figure 2.6B**). From the increased expansion of CD8 effector T cells elicited by the effects of IL-33 adjuvant properties, we can postulate their important role in providing tumor protection as shown in **Figure 6B**. Subsequently, we also demonstrate that a boost vaccination can further expand the formation of Ag-specific CD8 T cells after a prime vaccination suggesting the

potential recall of the established memory CD8 T cell pool (**Figure 2.8B**). The reasons behind the ability of IL-33 to expand the frequency of CD8 T cells are not yet entirely clear (12,13,41,45). However, further studies are needed to elucidate these mechanisms.

From a therapeutic point of view, the goal of successful vaccination is the induction of the most potent subsets of CD8 memory T cell populations to rapidly control infection. Recently, reports have begun to show that the effector-memory KLRG1⁺CD8⁺ T cell population can mediate potent protective immunity against certain pathogens (37-39) and might be optimal for immediate regression of established subcutaneous (s.c.) tumors (40). Mice immunized with IL-33 demonstrated robust expansion of activated effector memory CD8⁺ T cells in the periphery (**Figure 2.8C**), suggesting trafficking of activated CD8⁺ T cells to the site of Ag stimulation. Our findings support the concept that vaccine-induced effector-memory CD8 T cell responses might be important memory CD8⁺ T cell subsets for an effective therapeutic vaccine against tumors (40). The high frequency of Ag-specific effector-memory cells in the periphery is consistent with the observation that effector-memory T cells can migrate to the site of infection and initiate immediate effector function (46). Furthermore, these results are in agreement with Bonilla *et al.*, reporting IL-33 is important for primary effector CD8 T cell responses (12). However, they show that IL-33 may not play a role in memory responses, while our findings suggest that in certain cases it may play an important role. The reasons for the differences between the two studies are currently unknown, but may be due to differences in model systems. We also demonstrate that secondary memory cells after boost showed a greater formation of CD62L⁻KLRG1⁺ cells in the periphery (**Figure 2.8C**). Together, these results indicate that the increase in the frequency and phenotype of the IL-33-adjuvanted vaccine-induced Ag-specific P14 CD8 T cells after a prime and

boost vaccination may be a prediction of the protective correlates of immunity behind the therapeutic efficacy of immunoadjuvant IL-33 against the established TC-1 tumors (**Figure 2.6B**). We are currently investigating the ability of IL-33 to generate central memory immunity, since central memory T cells are important subsets of memory CD8 T cells that also mediate optimal protective immunity against pathogens (47,48). Overall, understanding the mechanism of action by which IL-33 influences the expansion and development of heterogeneous CD8 T cell populations in vaccines is an important area for further investigation. Altogether, these results support evidence that IL-33 acts as a potent adjuvant capable of inducing and modulating potent Ag-specific cell-mediated immunity in a variety of pathogens.

In summary, we provide insight into the biological function of proIL-33 and mtrIL-33 and its effects on modulating the adaptive immune responses *in vivo*, inducing potent Ag-specific anti-viral and anti-tumor T_H1 and CD8 T cell immunity, which resulted in effective tumor regression. This study provides evidence that immunoadjuvant IL-33 elicits its effects by enhancing the formation of the Ag-specific effector CD8 T cells and markedly amplifying the effector-memory CD8 T cells responses. These findings, we believe, establish the validity of IL-33 as a new adjuvant for consideration in the context of immune-therapies, in particular, for cancer vaccine therapies.

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CHAPTER 3

MOLECULAR ADJUVANT IL-33 ENHANCES THE POTENCY OF A DNA VACCINE IN A LETHAL CHALLENGE MODEL

ABSTRACT

Identifying new molecular adjuvants that elicit effective vaccine-induced CD8 T cell immunity may be critical for the elimination of many challenging diseases including Tuberculosis, HIV and cancer. Here, we report that co-administration of molecular adjuvant IL-33 during vaccination enhanced the magnitude and function of antigen (Ag)-specific CD8 T cells against a model Ag, LCMV NP target protein. These enhanced responses were characterized by higher frequencies of Ag-specific, polyfunctional CD8 T cells exhibiting cytotoxic characteristics. Importantly, these cells were capable of robust expansion upon Ag-specific restimulation *in vivo* and conferred remarkable protection against a high dose lethal LCMV challenge. In addition, we demonstrate the ability of IL-33 to amplifying the frequency of Ag-specific KLRG1⁺ effector CD8 T cells. These data show that IL-33 is a promising immunoadjuvant at improving T cell immunity in a vaccine setting and suggest further development and understanding of this molecular adjuvant for strategies against many obstinate infectious diseases and cancer.

INTRODUCTION

There is still a great need for effective vaccines against many chronic infectious including HIV, HCV, Tuberculosis and malaria. For these pathogens, it is known that T cell-mediated immunity is critical in either controlling, preventing or delaying the onset of disease (1). Thus, a crucial step in vaccine development for these infections requires producing cytotoxic T_H1 versus humoral T_H2 responses. Currently, licensed vaccines such as inactivated and recombinant protein or non-live vaccines predominately drive humoral immune responses (2). An overall shortcoming of these vaccines, especially non-live vaccines, is their inability to generate both effective T_H1 and CD8 T cell immunity, thus hindering their beneficial role in limiting or preventing diseases that require adaptive cellular immune responses (2,3). One way to improve the quality of immune responses during vaccination is to incorporate immunoadjuvants, which have been shown to help increase their T_H1 immune potency (2). However, it has been a challenge to discover immunoadjuvants that can amplify the induction of CD8⁺ T cell responses. Notably, IL-33 has emerged as a proinflammatory cytokine capable of promoting both potent T_H1 and cytotoxic CD8 T cell immunity (4,5,6). Therefore, IL-33 has great potential to act as a potent molecular adjuvant in vaccines designed to boost CD8⁺ T cell immune responses.

IL-33 is a member of the IL-1 cytokine family, which is released by necrotic cells or activated innate immune cells during trauma or infection (6,7). Therefore, it is considered to serve as the first line of defense against pathogens, by providing an endogenous “danger signal” that triggers inflammation and promotes cell-mediated immune response. Originally studied in the context of T_H2 immunity associated with inflammatory disorders (6,7), evidence has begun to unveil IL-33’s unappreciated ability to induce T_H1 and CD8 T cell-mediated immunity (4-6). We have recently reported that

IL-33 can act as novel immunoadjuvant to induce both potent T_H1 and effective CD8 T cell responses in an anti-tumor DNA vaccine (5). Here we expanded the scope of these initial studies to evaluate the capacity of IL-33 to serve as a vaccine adjuvant to enhance and modulate cell-mediated responses against various models of infection that require CD8 T responses.

In the present study, we use the well-studied lymphocytic choriomeningitis virus (LCMV) model to investigate IL-33's ability to facilitate the induction of antiviral and protective immunity and further elucidate its biological function on memory CD8 T cell expansion and differentiation in a vaccine setting. We hypothesize that IL-33 would have the capacity to improve the efficacy of DNA vaccines against a viral challenge, providing optimal effector function and protection. Here we show that the administration of IL-33 coadministered with a DNA vaccine against LCMV induces robust antigen-specific IFN γ responses, enhances antigen (Ag)-specific polyfunctional CD8⁺ T cell immune responses, increases the cytotoxic phenotype of the CD8 T cells, and provides substantial protective immunity against a high-dose lethal LCMV challenge. We also that inclusion of IL-33 can significantly amplify and expand the Ag-specific effector memory CD8 T cell responses. Furthermore, we provide evidence of IL-33's ability to also enhance cell-mediated immune responses when co-delivered with an HIV DNA vaccine. These findings significantly highlight the important role of IL-33 as a potential future vaccine adjuvant with applicability in the treatment of a variety of chronic viral diseases that require potent T_H1-type immunity for their prevention or control.

METHODS

Constructs

The DNA constructs encoding mature IL-33 (mtrIL-33), HIV (ConC) and LCMV-GP construct has been described (5-10). All constructs had highly efficient immunoglobulin E (IgE) leader sequence inserted at the 5'end of the gene. The constructs were commercially synthesized and optimized as described previously (10).

Animals

All animals were conducted and maintained in accordance with the NIH and the University of Pennsylvania Institutional Animal Care and Use Committee guidelines. Female C57BL/6 (H-2^b) 8-week-old mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Animal immunizations

Mice were immunized once intramuscularly (i.m.) in the tibialis anterior muscle. *In vivo* electroporation (EP) was delivered, with the CELLECTRA adaptive constant current EP device (Inovio Pharmaceuticals), at the same site immediately following vaccination as previously described (5,10). The mice were immunized with either 10 µg pVAX1 or 10 µg pLCMV-NP with or without 11 µg of mtrIL-33 construct. Three weeks after initial immunization, mice were sacrificed and splenocytes were harvested to measure immune responses. The LCMV-GP (GP) construct was administered at 10 µg. For the HIV immunizations, mice were immunized three times at two-week intervals with 10 µg of each construct (ConC) with or without 11 µg of mtrIL-33. One week after immunization, the mice were sacrificed and splenocytes were harvested to monitor immune responses. All studies were repeated at least two times.

LCMV Viral Challenge

For lethal challenge studies, immunized mice were challenged intracranial (i.c.) 21 days after initial vaccination with either 20xLD₅₀ or 40xLD₅₀ of LCMV Armstrong as previously described (11) in 30 µl of virus diluent (PBS with 20% FBS and 1X Anti-Anti (Invitrogen, Carlsbad,CA)). All mice LCMV challenged were housed in a BSL-2 facility and were observed daily for 21 days.

ELISPOT assay

For mice vaccinated with DNA all spleens were processed and IFN γ ELISpot assays were performed to determine the antigen-specific cytokine secretion. Spleens were collected in RPMI 1640 medium (supplemented with 10% FBS, 1X Antibiotic-Antimycotic, and 1X β -ME) and splenocytes were isolated by mechanical disruption of the spleen using a Stomacher machine (Seward Laboratory Systems, Bohemia, NY). The resulting mashed spleens were filtered using a 40µm cell strainer, treated with ACK lysis buffer for 5 minutes to lyse the RBCs, washed in PBS and then resuspended in RPMI medium for use in ELISpot or Flow Cytometry assay. The IFN γ ELISPOT assays were conducted as previously described in detail (5,10,11). The measurement of LCMV-specific T cell responses were assessed by stimulating splenocytes with immunodominant LCMV epitope from the H-2^b background (D^bNP₃₉₆₋₄₀₄ (NP396)) or (D^bGP₃₃₋₄₁ (GP33)) (Invitrogen). The HIV-specific T cell responses were measured by using pooled peptides (15-mers overlapping by 9 amino acids; 2.5 µg/ml final concentration). All peptides were synthesized from GenScript. Concavalin A (Sigma-Aldrich, St. Louis, MO) was used as positive control and complete culture medium was

used as negative control. Spots were enumerated using an automated ELISPOT reader (Cellular Technology, Shaker Heights, OH).

Flow cytometry

Lymphocytes were isolated and processed from the peripheral blood as previously described (5). Cells were stained with CD8, CD44, CD62L, KLRG1, and MHC class I peptide tetramer to LCMV-GP33 (KAVYNFATC) (Beckman Coulter) as described previously (5). Intracellular cytokine staining was performed after 5 hr of ex vivo stimulation with either LCMV epitope D^bNP₃₉₆₋₄₀₄ or D^bGP₃₃₋₄₁ peptide, HIV and Ag85B pooled peptides depending on the study as described (5,10). The following antibodies were used for surface staining: LIVE/DEAD Fixable Violet Dead Cell stain kit (Invitrogen), CD4 (FITC; clone RM4-5; ebioscience), CD8 (APC-Cy7; clone 53-6.7; BD Biosciences); CD44 (A700; clone IM7; Biolegend). For intracellular staining the following antibodies were used: IFN γ (APC; clone XMG1.2; Biolegend), TNF α (PE; clone MP6-XT22; ebioscience), CD3 (PerCP/Cy5.5; clone 145-2C11; Biolegend); IL-2 (PeCy7; clone JES6-SH4; ebioscience). All data was collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR) and SPICE v5.2 (free available from <http://exon.niaid.nih.gov/spice/>). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals.

Bone Marrow Dendritic Cell Maturation

Bone marrow was harvested from femurs and tibias of female C57BL/6 mice age 6-8 weeks. Following RBC lysis, bone marrow cells were cultured in 40 ng/mL recombinant

murine GM-CSF and incubated at 37° C, 5% CO₂. Media was refreshed on days 3 and 6. On day 8, immature BMDCs were harvested by pipetting floating and loosely adherent cells. BMDCs were subsequently treated for 24 hours with 100 ng/mL recombinant IL-33 or vehicle-sham (PBS) control. Following treatment, BMDCs were harvested by scraping and analyzed by flow cytometry against CD40, CD80, CD86, or isotype-matched control antibodies.

Luminex

Cytokines and chemokines were quantified in supernatant collected from BMDC treated with or without 100ng/ml of rIL-33 (R&D Systems) at 24 hours, using a custom Millipore cytokine assay (Millipore, Billerica, MA) according to manufacturer's instructions. The panel of cytokines and chemokines included: IL-1a, IL-1b, IL-2, IL-6, IL-12, TNFa, IP-10, MIG and RANTES. Samples were read on a Bio-Plex 200 system with Bio-Plex Manager Software (Bio-Rad).

T cell proliferation assay

Splenocytes isolated from immunized B6 mice 21 days after initial immunization were labeled with Cell Tracer violet Violet (Molecular Probes) and pulsed with 10 µM peptide for 5 days. CD8 T cell proliferation was determined using flow cytometry to assess Cell Trace Violet dilution.

Statistical Analysis

Group analyses were completed by matched, two-tailed, unpaired *t*-test and survival curves were analyzed by log-rank Mantel-Cox test. For non-equally distributed samples nonparametric Mann-Whitney test was performed (**Figures 3.2E and 3.2F**). All values

are mean \pm SEM and statistical analyses were performed by GraphPad Prism (La Jolla, CA).

RESULTS

IL-33 elicits protection against a lethal LCMV challenge

The LCMV infection model has been extensively used to understand the role of virus-specific CD8⁺ T cell responses in the context of vaccine-elicited protection (12-14). Considering our recent novel finding that IL-33 can act as an immunoadjuvant to induce both anti-viral and anti-tumor CD8 T cell immunity (5), we used the intracranial (i.c.) LCMV challenge model to further study the protective efficacy of IL-33. Three groups of C57BL/6 mice (B6) (n = 10) were vaccinated by electroporation (EP) one time with 10 µg of pLCMV-NP (NP) construct with or without 11 µg of mature IL-33 (mtrIL-33) construct. The empty vector pVAX was used as a negative control. The LCMV NP structural protein is recognized as a critical component and target for protective LCMV immunity since it is not a target for neutralizing antibodies (11). All animals were challenged 21 days post-vaccination (dpv) with a lethal 20xLD₅₀ dose of LCMV Armstrong (**Figure 3.1a**) (11,14-16). Vaccinated animals with NP plus mtrIL-33 showed complete protection while the NP alone group achieved only 60% protection (**Figure 3.1b**). On the other hand, all control pVAX vaccinated animals succumbed to infection. After showing that mice immunized using mtrIL-33 as an adjuvant exhibited 100% survival rate, we next sought to determine whether vaccinated mice with adjuvant could confer protection against an even higher lethal dose of LCMV challenge. Therefore, mice were challenged with a 40xLD₅₀ dose of LCMV Armstrong (11), 21 days post-single vaccination (**Figure 3.1c**). Notably, animals receiving one immunization of NP plus mtrIL-33 yielded a significant 80% protection, while the NP alone group only conferred 10% protection against this highly lethal dose of LCMV (**Figure 3.1c**). These data show that IL-33 elicits protection against a lethal LCMV challenge.

IL-33 significantly increases LCMV-specific CD8 T cell responses

Considering that CD8 T cell responses are essential for facilitating control against LCMV (11-16), we hypothesized that the IL-33 adjuvanted vaccine induced CD8 T cells mediated antiviral protection. Thus, to better characterize the protective immune correlates driven by mtrIL-33, groups of mice ($n = 4-5$) were immunized once with NP either with or without mtrIL-33. The magnitude of NP-specific immune responses was measured 21 dpv in response to peptide re-stimulation (2.5 $\mu\text{g/ml}$) using the immunodominant epitope in the H-2^b background: D^bNP₃₉₆₋₄₀ (NP396) (11). Compared to NP alone-vaccinated mice, we found that co-immunization with mtrIL-33-encoding plasmid elicited stronger NP-specific T cell responses by greater than 2.5 fold (**Figure 3.2a**); IFN- γ ELISPOT counts were $\sim 2,500$ spot-forming cells [SFCs] per 10^6 splenocytes in the IL-33 vaccinated mice versus ~ 980 SFC/ 10^6 splenocytes for the NP alone group. Next, we assessed the phenotypic and functional profile of vaccine-induced CD8⁺ T cells in response to NP396 peptide re-stimulation (2.5 $\mu\text{g/ml}$ final peptide concentration). Twenty-one days after vaccination there was a significant difference among vaccine groups in the frequency of CD8 T cells producing effector cytokines (**Figures 3.2b,c**). The NP vaccine coadministered with mtrIL-33 elicited a higher percentage of Ag-specific CD8 T cells producing all three cytokines (**Figure 3.2b**), and a significant number of the CD8 T cells were polyfunctional (**Figure 3.2c**). Compared with the NP alone vaccinated group, the NP+mtrIL-33 vaccinated group elicited substantially higher frequencies of NP-specific CD8 T cells producing either IFN γ alone (NP, 1.3%; NP+mtrIL-33, 2.3%), dual IFN γ ⁺TNF α ⁺ (NP, 0.76%; NP+mtrIL-33, 1.63%), or triple-positive IFN γ ⁺TNF α ⁺IL-2⁺ (NP, 0.20%; NP+mtrIL-33, 0.43%) in the spleens 21 dpv (**Figure 3.2c**). Collectively, the enhanced Ag-specific CD8⁺ T cell response induced by IL-33 is indicative of IL-33's

ability to provide substantial protection against LCMV challenge. We next characterized the cytotoxic potential of vaccine-induced CD8 T cells. CD8 T cells isolated from mice vaccinated with IL-33 showed a significantly higher frequency of antigen-specific (IFN γ ⁺CD107a⁺: 2.5%) degranulation compared to NP alone-vaccinated mice (IFN γ ⁺CD107a⁺: 1.2%) (**Figure 3.2d**). We next evaluated the proliferative capacity of the CD8 T cells by monitoring Cell Trace Violet dilution in splenocytes isolated from mice 21 dpv rechallenged *in vitro* with NP396 peptide re-stimulation. **Figure 3.2e** shows that mtrIL-33 vaccinated mice underwent significantly higher Ag-specific proliferation of CD8 T cells, being ~2 fold greater than NP control group. Notably, there was an enrichment of effector memory CD8 T cells (CD44⁺CD62L⁻) in the adjuvant-vaccinated group (**Figure 3.2f**). There was no significant difference in the central memory CD8 T cell (CD44⁺CD62L⁺) population (data not shown). Taken together, the inclusion of IL-33 elicits robust levels of NP-specific T cell immunity, especially enhancing CD8 T cell immune responses.

To better understand the biological function of IL-33 on the induction of Ag-specific CD8⁺ T cells during the course of vaccination, we further characterized IL-33's ability to expand the Ag-specific effector memory CD8 T cell population. To achieve this goal, we took advantage of the well-studied D^bGP₃₃₋₄₁ MHC class I tetramer to follow Ag-specific CD8 T cells as they develop after initial priming. Mice were vaccinated once with a LCMV glycoprotein LCMV-GP (GP) DNA vaccine and the frequency of D^bGP33-specific CD8⁺ T cells was monitored in the peripheral blood during the course of vaccination either with or without mtrIL-33 (**Figure 3.4a**). Delivery of IL-33 expanded the number of D^bGP33 tetramer-specific CD8 T cells in the peripheral blood (**Figure 3.3a**). In peripheral blood lymphocytes (PBLs), the frequency of GP33-specific CD8 T cell was

significantly 2-fold higher at 18 and 21 dpv compared with the nonadjuvanted group (**Figure 3.3a**). Similarly, the inclusion of IL-33 also increased the number of GP33-specific CD8⁺ T cells in the spleen 21 dpv (**Figure 3.3b**) and the Ag-specific CD8 T cells secreting IFN γ , undergoing degranulation, and expressing the transcription factor T-bet (**Figures 3.3c-f**). Additionally, all mice were boosted with the GP construct alone (21 days after initial immunization) to quantify the Ag-specific recall responses. Compared to control group, the IL-33 vaccinated group significantly increased the Ag-specific CD8⁺ T cells. Notably, the IL-33 immunized group GP33 tetramer-specific T cells were ~3-fold higher starting 3 days post-boost vaccination (d24) compared to the NP-vaccinated group (**Figure 3.3a**). The significant difference in the amplification of the GP33-specific CD8 T cells was still observed 10 days after DNA boost (d31). Consistent with **figure 3.2**, the current data further confirms IL-33's ability to induce the quality of Ag-specific CD8 T cells which seemed to correlate with IL-33's adjuvant effect to mediate its antiviral protection as shown in **Figure 3.1**. Finally, since it has been demonstrated that effector-phenotype memory CD8 T cells (T_{eff}) can mediate clearance of blood-borne pathogens (17-22), we next evaluated the ability of IL-33 to induce the differentiation of T_{eff} cells based on expression markers: CD44 and KLRG1 (**Figure 3.4b**). The administration of mtrIL-33 resulted in a significant expansion in the percentages of CD8⁺KLRG1⁺ T_{eff} cells in the PBLs, compared with the GP-only vaccinated group (**Figure 3.4c**). We also evaluated the recall response of D^bGP33 tetramer-specific CD8⁺KLRG1⁺ T cells after DNA-GP boosting, 21 days after initial immunization and observed marked expansion of GP33-specific memory CD8⁺KLRG1⁺ T cells in both groups after boosting; however, the proportion of CD8⁺KLRG1⁺ T cells remained significantly higher in the mtrIL-33 adjuvant group (**Figure 3.4c**). In summary, IL-33 significantly increased LCMV-specific CD8 T cell

immunity against two separate viral proteins and enhanced effector-memory CD8⁺KLRG1⁺ T cell subset differentiation.

IL-33 promotes DC maturation in vitro

It is well known that antigen presented by activated DCs and the type of production of polarizing cytokines they secrete can promote different fates on T cell development (23). We next investigated whether IL-33 could induce DC maturation by assessing the up-regulation of certain surface expression markers and their induction of proinflammatory cytokines, all of which may influence adaptive immunity. Mouse bone marrow-derived dendritic cells (BMDC) incubated with 100 ng/ml of recombinant IL-33 (rIL-33) for 24 hours up-regulated the expression of co-stimulatory CD80 and CD86 and CD83 (marker for DC maturation) molecules (**Figure 3.5a**). Moreover, to characterize the cytokines induced by the effect of IL-33, we employed a multiplex cytokine array consisting of a panel of T_H1 cytokines (IL-12), T_H2 cytokines (IL-4), anti-inflammatory cytokines (IL-10), and pro-inflammatory cytokines (IL-1a, IL-1b, IL-6, TNF- α). In addition, we included cytokines and chemokines associated with activation and chemoattraction of T cells: RANTES (CCL5), IP-10 (CXCL10) and MIG (CXCL9). As shown in **figure 3.5b**, rIL-33 stimulated DC production of a variety of proinflammatory cytokines and chemokines. Additionally, DCs incubated with rIL-33 showed no detectable production anti-inflammatory suppressive cytokine IL-10 and of the prototypical T_H2 cytokine IL-4 (data not shown). Therefore, the biological function of IL-33 exhibits the capacity to induce phenotypic maturation of DCs, which have the potential to drive proinflammatory cytokines and chemokines that may facilitate a polarized T_H1/CD8⁺ T cell protective response.

IL-33 augments HIV-specific T cell-mediated responses

To determine whether mtrIL-33 adjuvant can enhance the vaccine potency for other pathogens requiring both T_H1 and CD8 T cell responses, we assessed the Ag-specific T cell-mediated responses of mtrIL-33 co-delivered with a HIV DNA vaccine antigen (**Figure 6.6**). B6 mice ($n = 5$) were vaccinated three times i.m. at two week intervals with 10 μ g HIV Consensus clade C (ConC) alone or in combination with 11 μ g of mtrIL-33 followed by EP. One week after final immunization Ag-specific immune responses were measured using cells derived from the spleen. Consistent with findings in the LCMV model (**Figures 3.2a-c**), the inclusion of mtrIL-33 enhanced the numbers of HIV-specific $IFN\gamma$ secreting T cells (ConC, ~3,800 SFC) when compared with non-adjuvanted groups (ConC, ~2,300 SFC) (**Figure 3.6a**). Furthermore, we characterized the cytokine-producing phenotype of the T cell population after immunization with the HIV DNA (**Figures 3.5b,c**). In mice, the Ag-specific T_H1 response after HIV vaccination with mtrIL-33, consisted of significantly high frequency of polyfunctional double-positive ($IFN\gamma^+TNF\alpha^+$) and $TNF\alpha^-$ and $IFN\gamma$ -single-positive CD8 T cells in the spleen (**Figure 3.6B**). Regarding CD4 T cells, the Ag-specific T_H1 response after HIV plus IL-33 vaccination, consisted of high frequency of polyfunctional triple-positive ($IFN\gamma^+TNF\alpha^+IL-2^+$), double-positive ($IFN\gamma^+TNF\alpha^+$) and $IFN\gamma$ single-positive CD4 T cells in the spleen (**Figure 3.6C**). Overall, these findings significantly highlight the important role of IL-33 as an effective immunoadjuvant to be incorporated into future vaccines targeting an array of microbial infections.

FIGURES

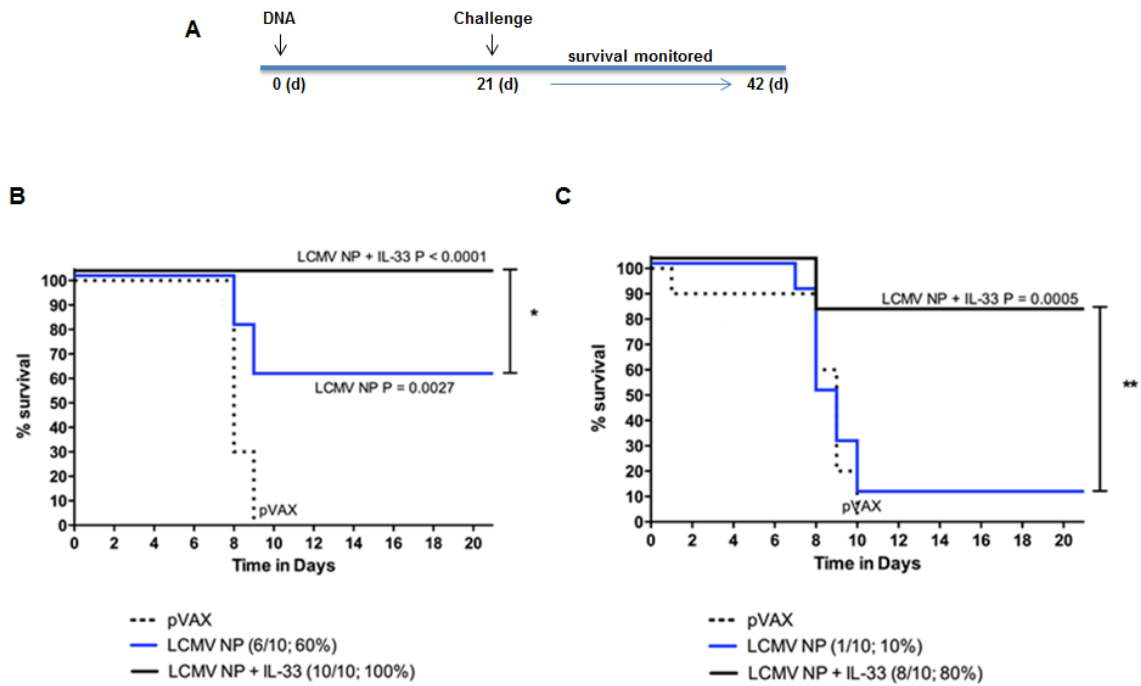


Figure 3.1. IL-33-elicited protection against lethal LCMV challenge. (a) Mice ($n = 10/\text{group}$) were immunized one time i.m. using EP with $10\ \mu\text{g}$ of empty vector control plasmid (pVAX) or $10\ \mu\text{g}$ of LCMV-NP with or without $11\ \mu\text{g}$ of mtrIL-33. At day 21 post-vaccination, mice were either challenged intracranial (i.c.) with (b) $20\times\text{LD}_{50}$ armstrong LCMV or (c) $40\times\text{LD}_{50}$ armstrong LCMV and animal survival was monitored for 21 days post challenge. Experiments were performed at least two times in independent experiments and data are representative of the result. *, $P < 0.05$; **, $P < 0.01$.

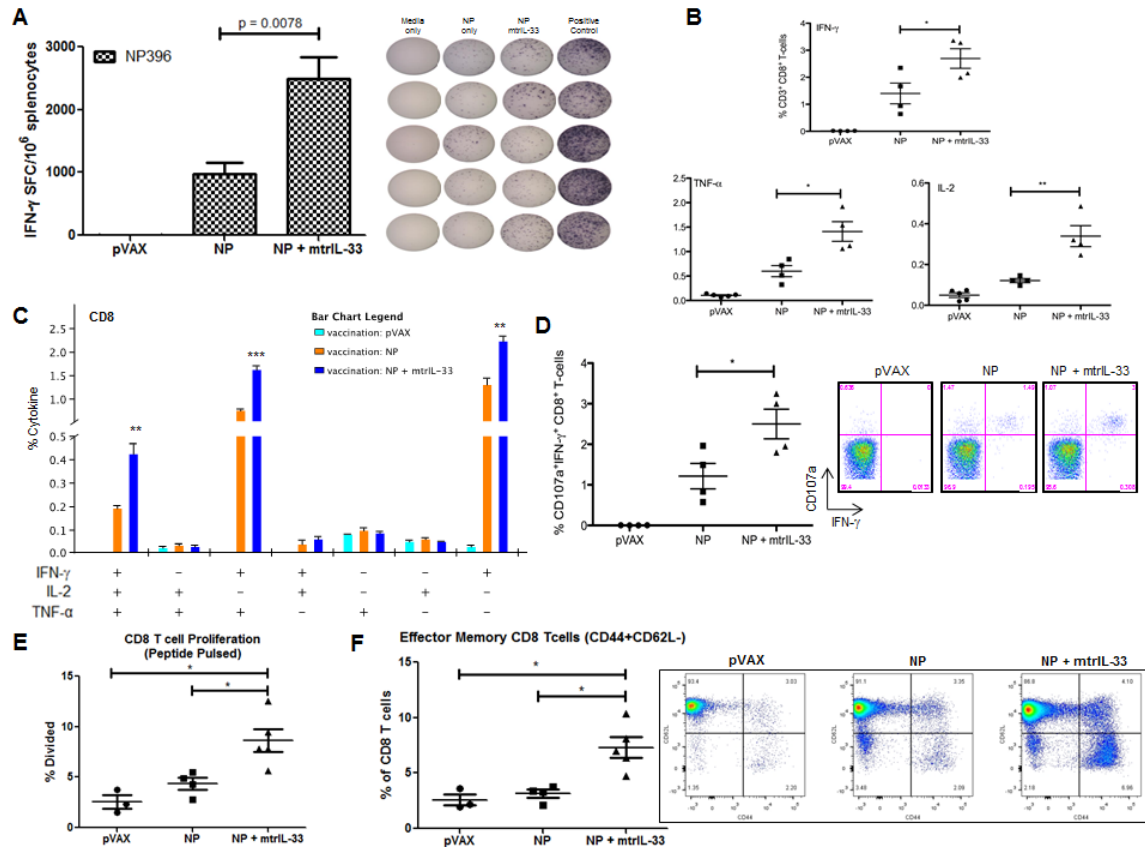


Figure 3.2. IL-33 induces potent antigen-specific effector CD8 T cells. C57BL/6 mice ($n = 4-5$) were immunized once by i.m. followed by EP with 10 μg of NP with or without 11 μg of mtrIL-33. Splenocytes were harvested 21 days post vaccination to assess the cellular immune responses. ELISpot and ICS assays were stimulated for 18 hours and 5 hours, respectively, with a final concentration of 2.5 $\mu\text{g}/\text{ml}$. Mice that received 10 μg pVAX only served as a negative control. (A) The ability of T cells to produce IFN γ in response to D^bNP396 epitope (CD8) was determined by IFN γ ELISpot assay. (B) Flow cytometry was used to determine the total cytokine (IFN γ , TNF α and IL-2) frequencies of the D^bNP396-specific CD8 T cells induced by mtrIL-33. (C) Column graph shows multifunctional subpopulations of single-, double- and triple-positive CD8 T cells releasing the cytokines IFN γ , TNF α and IL-2. (D) The antigen-specific cytolytic degranulation T cells induced by mtrIL-33 adjuvant were measured by degranulation marker expression, CD107a and producing IFN γ . Dot plots show representative examples of each mouse group. (E) Splenocytes from mice were labeled with cell tracer violet and stimulated with D^bNP396 peptide. Cell tracer signals on CD8 T cell population were detected by flow cytometry to measure proliferation. (F) After proliferation the % of effector CD8 T cells upon stimulation with NPD^b396-specific peptide (2.5 $\mu\text{g}/\text{ml}$) was assessed. Dot plots show representative figure of CD62L⁻CD44⁺ CD8 T cells after stimulation. Data shows the SEM of two independent experiments repeated at least two to three times. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with NP.

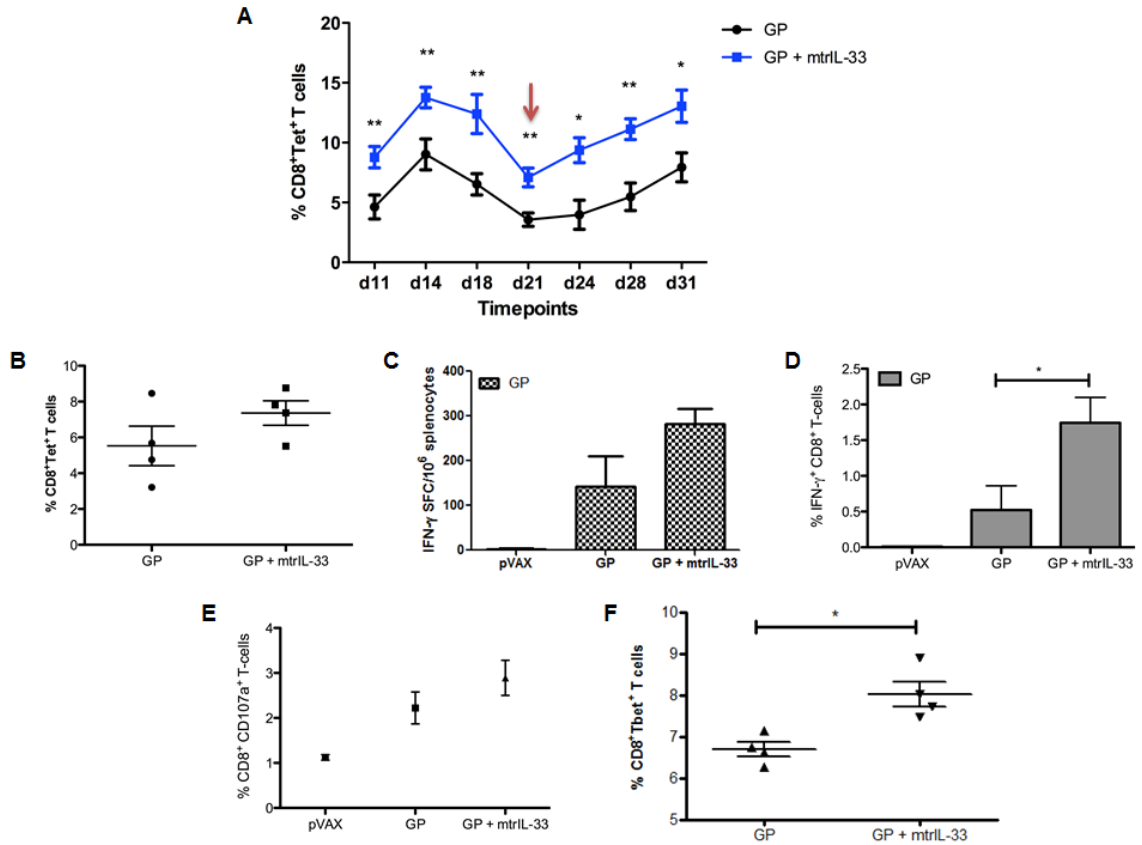


Figure 3.3. IL-33 amplifies the percentage of antigen-specific CD8 T cells. C57BL/6 mice ($n = 4-8$) were immunized once with $10 \mu\text{g}$ LCMV-GP (GP) construct with or without mtrIL-33 and the Ag-specific CD8 T cell population was monitored during the course of vaccination. (A) Kinetics $\text{D}^b\text{Gp33}(\text{Tet}^+)$ -specific CD8 T cells in the blood following DNA vaccination with a prime at day 0 and boost with GP alone (red arrow) on day 21 post vaccination (dpv). The cells are gated on live $\text{CD8}^+\text{CD44}^+$ T cells. (B-F) At day 21, spleens ($n = 4$ per group) were harvested and antigen-specific responses were monitored ex vivo with GP33 peptide. (B) Frequency of $\text{D}^b\text{GP33}$ CD8^+ T cells at 21 dpv. (C) IFN- γ ELISpot assay used to measure the GP-specific T cells producing IFN- γ on 21 dpv. (D) Frequency of GP-specific IFN- γ^+ CD8^+ , (E) $\text{CD107a}^+\text{CD8}^+$, and (F) $\text{Tbet}^+\text{CD8}^+$ T cells at 21 dpv. Data shows the SEM of two independent experiments repeated at least two times. *, $P < 0.05$; **, $P < 0.01$; no statistical difference in Fig 3 B, C, E.

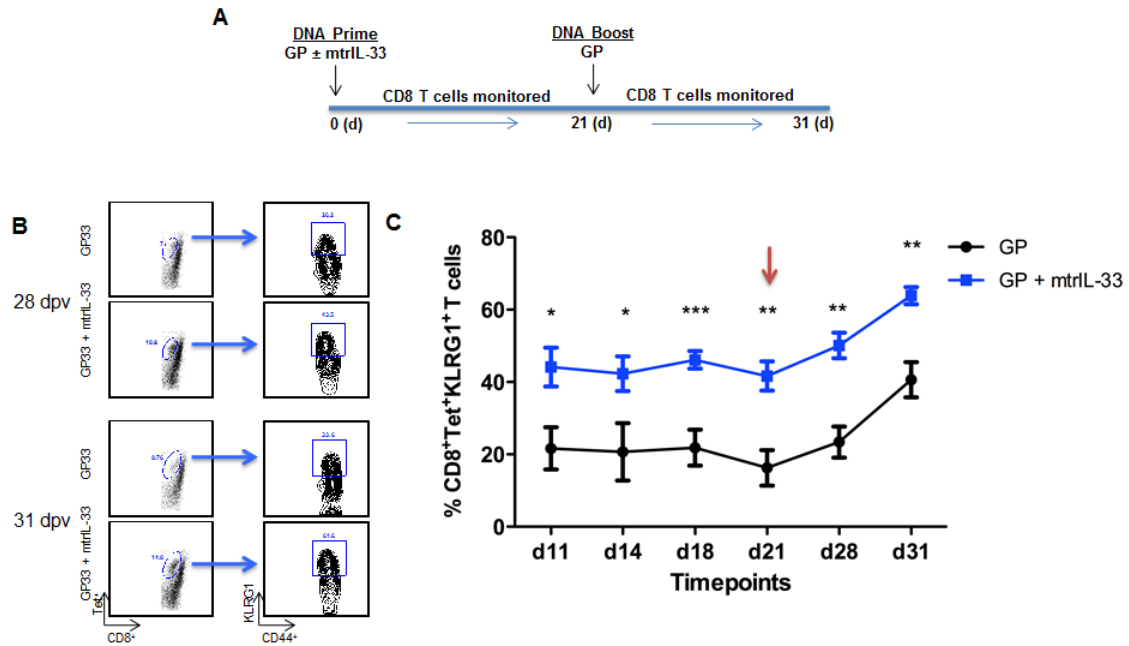


Figure 3.4. IL-33 enhances the expansion of CD8⁺KLRG1⁺ effector-memory T cells. (A) Vaccine immunization schedule in mice. B6 mice ($n = 4-8$) were immunized once with 10 μg of GP plasmid with or without 11 μg of mtrIL-33 construct and boosted with only GP (red arrow) at 21 days after initial vaccination. Ag-specific responses in the blood were monitored as indicated. (B) Gating strategy used to identify the D^bGp33⁺CD44⁺KLRG1⁺CD8⁺ T cell population in the blood following vaccination (C) Kinetics of the CD8⁺KLRG1⁺ T cell population after DNA vaccination. Experiments were repeated two times. Population were gated on D^bGp33⁺CD8⁺CD44⁺KLRG1⁺ *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

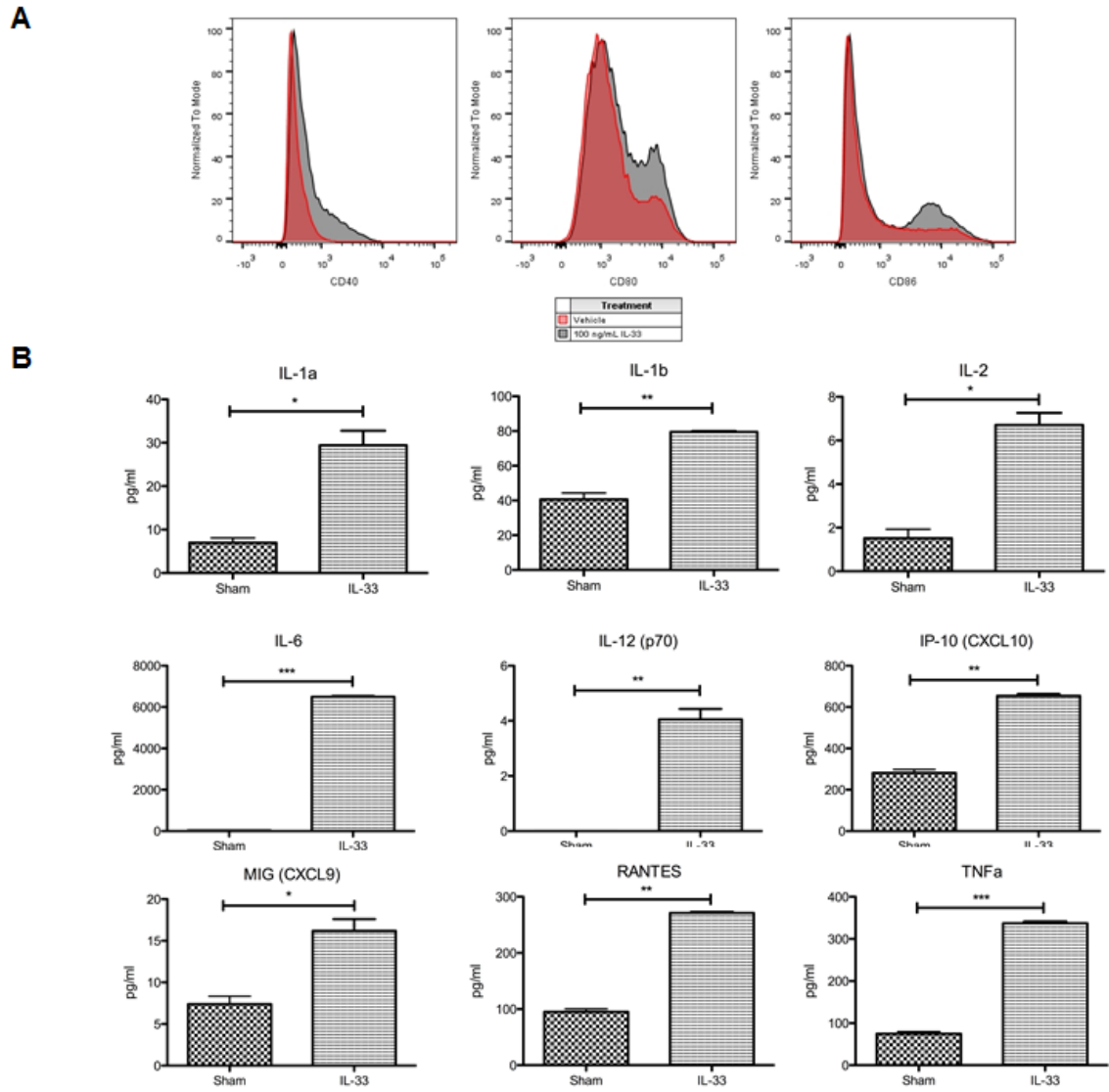


Figure 3.5. IL-33 promotes phenotypic and functional maturation of DCs. (A) Mouse bone marrow dendritic cells (BMDCs) were incubated in medium in the absence (sham-PBS) or presence of 100 ng/ml recombinant IL-33 (rIL-33) for 24 hours before measurement of the indicated DC surface marker expression by flow cytometry (red, isotype-matched control). The data shown represent the results of one experiment repeated twice. (B) Supernatants were obtained after BMDCs were incubated in the absence or presence of 100 ng/ml for the measurements of the indicated cytokines. Shown is the SEM of duplicate wells of one experiment representative of two. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

DISCUSSION

Interleukin 33 is a pleiotropic cytokine whose properties extend beyond T_H2 responses. We show here that IL-33 is capable of inducing and modulating T_H1 responses (6). However, the biological role of IL-33 as an adjuvant in vaccines remains to be further elucidated. The mature form of IL-33 delivered as an adjuvant remarkably augments LCMV-specific cell-mediated antiviral immunity and provides substantial protection against a high-dose lethal LCMV challenge.

The LCMV model of infection is well established with great applicability to investigations regarding vaccine-induced immunity. In a previous study, we showed that mice immunized once with a LCMV-NP DNA vaccine yielded 67% protection after a 20xLD₅₀ lethal dose of LCMV challenge (11). Therefore, we sought to investigate whether the inclusion of IL-33 after one immunization would increase its protective efficacy since a high frequency of LCMV-specific CD8⁺ T cells is considered a critical characteristic against an LCMV infection (12-16). Here, the coadministration of IL-33 not only increased the magnitude of IFN γ spot-forming NP396-specific CD8 T cells, but also improved their polyfunctionality, increased the cytolytic phenotype of the CD8 T cells, and their effector memory differentiation. As measured by IFN γ ELISpot for the specific CD8 T cell epitope D^bNP₃₉₆₋₄₀, the inclusion of IL-33 induced a 2.5-fold greater response compared to NP-alone immunization. IL-33 also induced greater responses compared with our previous LCMV-NP DNA vaccine study, where 25 μ g of pLCMV-NP administered i.m. twice at two week intervals only elicited ~1400 SCFs per 10⁶ splenocytes (11). Nevertheless, although our IL-33 adjuvant approach matched the immunogenicity of the 35 μ g of LCMV-NP dose that elicited 100% protection eventually after three immunizations (11), our IL-33-assisted DNA vaccination conferred 100%

protection only after one immunization (**Figure 3.1**). Furthermore, we show that IL-33 enhanced the polyfunctional CD8⁺ T cell populations secreting IFN γ ⁺TNF α ⁺IL2⁺, IFN γ ⁺TNF α ⁺ and IFN γ ⁺ and elicited a greater Ag-specific CD8 cytolytic degranulation (**Figure 3.2**). We find this data in accordance with our previous findings that IL-33 can increase the Ag-specific cell-mediated immune response when coadministered with a DNA vaccine (5) and with others (4,6). The reasons behind the role of IL-33 to enhance the frequency of CD8 T cells are not entirely clear. Nevertheless, recently Luzina et al. have shown in a vaccine delivery approach that mature IL-33 (the same isoform used in our vaccine) effect is ST2-dependent (24). Moreover, substantial research has shown the ST2 is important for IL-33 action as mice deficient in ST2 are entirely unresponsive to IL-33 (25). Therefore, the ability of CD8 T cells to respond to IL-33 is a possible explanation for the augmentation, as activated differentiated CD8 T cells can upregulate their cognate binding receptor ST2 (4,26) and therefore lead to more memory cells capable of responding to rechallenge. More importantly, consistent with the markedly enhanced cytotoxic CD8 T cell activity and the higher levels of NP396-specific effector memory CD8 T cells, the challenge studies using two different lethal doses of LCMV (**Figure 3.1**) validate IL-33's potent adjuvant properties, ultimately to improving antiviral vaccine efficacy (4,5).

The inclusion of adjuvants in vaccine formulations has long been a method of improving vaccine efficacy, but how adjuvants alter the phenotype and differentiation of T cells still remain unknown. Current studies have shown that the characteristic profiles of Ag-specific T cell responses can be correlated with superior disease protection (18,19,27-29). Therefore, to examine the biological function of mtrIL-33 to expand and differentiate the CD8 T cells, we coadministered mtrIL-33 with an LCMV-GP DNA

vaccine to monitor the D^bGP33 tetramer-specific T cells after immunization. In accordance with our previous data (5), we show that mtrIL-33, similar to full-length IL-33, can significantly amplify GP33⁺CD8⁺ T cell responses in the peripheral blood in a vaccine setting. Nevertheless, although not significant, a similar trend was observed whereby the frequency of GP33⁺CD8⁺ T cells in the spleen was higher in the IL-33 vaccinated group (**Figure 3.3B**). These results substantiated IL-33's cytokine property to mediate the expansion of CD8 T cells. In addition, we show that IL-33 also induced significant expansion of Ag-specific CD8⁺KLRG1⁺ T cells in the periphery (**Figure 3.4c**), as previously reported (4,5). These results can be explained by the ability of IL-33 to induce the trafficking of effector–memory T cells out of the spleen and into the periphery (**Figure 3.4c**), migrating to the site of infection to initiate immediate effector function (30–32) and protective immunity as illustrated in **figure 3.1**. Moreover, the significant higher recall responses of the Ag-specific CD8 T cells 8 days after boosting (**Figures 3.3a and 3.4c**) was due to higher basal levels of responses which likely correlated with IL-33's adjuvant effect. We find these results in accordance with Olson et al demonstrating that this population can expand after boosting (18). These findings further support the notion that effector-memory CD8 T cell responses can mediate potent protective immunity against certain pathogens (18,19). CD8⁺KLRG1⁺ T cells are usually considered as short-lived effector cells (22), and together, with recent reports demonstrating vaccines eliciting persistent effector-memory CD8 T cells may be essential for developing effective viral and cancer vaccines (27–29), suggest IL-33 as a suitable adjuvant for therapeutic vaccines. However, IL-33's role as a suitable adjuvant for preventative vaccines cannot be negated. On the contrary, new reports have suggested that KLRG1 expressing CD8 T cells may be capable of surviving long-term (18,19,30). And given that in this study IL-33 was administered in a prophylactic setting may also qualify IL-33's

utility as a preventative vaccine adjuvant. Nevertheless, because preventive vaccines should induce long-lasting Ag-specific responses, we are currently pursuing the role of IL-33 to induce central memory CD8 T cells.

One molecular mechanism behind IL-33's potential to drive the formation and differentiation of effector memory CD8 T cells is likely due to its ability to significantly increase T-bet expression in CD8 T cells *in vivo* (**Figure 3.3f**). Joshi and colleagues have shown that overexpression of T-bet is enough to induce the formation of KLRG1⁺ effector cells and that CD8 T cells lacking T-bet are impaired in forming these cytolytic effector cells (32). Therefore, our data further support the notion that an increase in T-bet is associated with effector CD8 T cell differentiation and their ability to have CTL phenotype. This finding also supports previous data showing that *in vitro* IL-33 can increase T-bet expression (26). Binfeng Lu and colleagues also reported that IL-33 promoted the effector CD8 T cells synergistically with TCR and IL-12 signaling (26). Therefore, given that the expression of T-bet in T cells is known to be induced by a combination of T-cell receptor and IFN γ signaling, could explain one underlining molecular mechanism by which IL-33 promotes effector-memory CD8 T cells (22,32,33). Nevertheless, further studies are needed to further elucidate these mechanisms and how IL-33 alters the transcriptional programs to induce memory T cell differentiation.

Interestingly, IL-33 was also associated with inducing the chemokines, CXCL9 (MIG) and CXCL10 (IP-10). These two chemokines have been reported to play a critical role in (1) recruiting T cells into the inflammatory sites, (2) vaccine-induced T-cell infiltration into the tumor, and (3) driving effective T_H1/CD8 T cell responses (34-43). To the best of our knowledge, this is the first time these two cytokines have been reported to be associated with IL-33. This provide further insight to one of the many potential

roles behind IL-33's ability to elicit robust effector T cell generation and trafficking in the periphery that may have correlated with IL-33's protective role in viral immunity as established in this study. Furthermore, this provides further insight to our previous finding on how IL-33's may have induced complete tumor regression in a therapeutic HPV-tumor model (5). In fact, MIG and IP-10 have been shown to inhibit tumor growth by recruiting effector T cells to tumors (44-46). Therefore, these cytokines critical roles for attracting T-cells to the site of infection, may explain IL-33's functional role on the impact of CD8 T cells observed in our study, recruiting the right type of immune cell infiltration necessary to facilitate viral immunity. We are currently investigating IL-33 roles on DC maturation and function during vaccination *in vivo*. Indeed, this data prompts further questions about how IL-33 acts on innate and adaptive immunity and how it bridges both arms of the immune system *in vivo*.

The goal of incorporating immunoadjuvants in vaccines is to regulate, modulate, and enhance the desired adaptive responses to prevent disease acquisition and/or progression. Therefore, understanding the biological functions and immunological mechanisms of immunoadjuvants can allow us to better understand correlates of vaccine immunogenicity, ultimately resulting in the design of better vaccines. Here we demonstrate that the administration of IL-33 serves as potent adjuvant improving vaccine efficacy by augmenting the frequencies of effective Ag-specific CD8 T cells, ultimately leading to significant antiviral protection in a widely used challenge model. This report also reveals new critical insight into the biological properties of IL-33 to perpetuate the differentiation of Ag-specific memory CD8 T cells during vaccination. Consideration of these findings may facilitate the development of better vaccine as well as to improve immune responses against cancer.

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CHAPTER 4

ALARMIN IL-33 ELICITS POTENT TB-SPECIFIC CELL-MEDIATED RESPONSES

ABSTRACT

Tuberculosis (TB) still remains a major public health issue despite the current available vaccine for TB, Bacille Calmette Guerin (BCG). An effective vaccine against TB remains a top priority in the fight against this pandemic bacterial infection. Adequate protection against TB is associated with the development of T_H1-type and CD8 T cell responses. One alarmin cytokine, interleukin 33 (IL-33), has now been implicated in the development of both CD4 T_H1 and CD8 T cell immunity. In this study, we determined whether the administration of IL-33 as an adjuvant, encoded in a DNA plasmid, could enhance the immunogenicity of a TB DNA vaccine. We report that the co-immunization of IL-33 with a DNA vaccine expressing the *Mycobacterium Tuberculosis* (Mtb) antigen 85B (Ag85B) induced robust Ag85B-specific IFN γ responses by ELISpot compared to Ag85B alone. Furthermore, these enhanced responses were characterized by higher frequencies of Ag85B-specific, multifunctional CD4 and CD8 T cells. Vaccination with IL-33 also increased the ability of the Ag85B-specific CD8 T cells to undergo degranulation and to secrete IFN γ and TNF α cytokines. These finding highlights IL-33 as a promising adjuvant to significantly improve the immunogenicity of TB DNA vaccines and support further study of this effective vaccine strategy against TB.

INTRODUCTION

Tuberculosis (TB) continues to be one of the most devastating infectious diseases existing worldwide and a major threat to global health. The causative agent of TB, *Mycobacterium tuberculosis* (Mtb), infects as many as 8.8 million new individuals per year with active TB and is responsible for over 1.4 million deaths annually (1). Against this threat, the BCG vaccine remains the only currently licensed TB vaccine approved for human use. While BCG has shown to have adequate efficacy against pediatric forms of TB, a major drawback has been its inability to protect against adult pulmonary TB (2,3). The current lack of an effective vaccine necessitates the urgent development of novel vaccine strategies against TB.

Recently, DNA-based vaccines have shown promise as a therapeutic platform for treating established HPV infections, because of their ability to evoke both humoral and cell-mediated immunity (4). Furthermore, the delivery of DNA vaccines, along with molecular cytokine adjuvants, by electroporation has greatly improved the effectiveness of DNA vaccines (5). Together these features make DNA vaccines an ideal approach for the development of an efficacious TB vaccine. In the past several decades only a few adjuvants have been approved for human use; however, these adjuvants mainly induce humoral immunity and CD4 T cell responses with relatively little CD8 T cell responses (6,7). Given the trending evidence that an important feature for an effective vaccine against Mtb will require both CD4 T_H1 and CD8 T cell responses (8-10), it will be imperative that new vaccine adjuvants tested induce optimal activation of both CD4 and CD8 T cells.

Alarmins are a unique group of endogenous molecules that initiate host defenses by inducing inflammation, activating wound healing, and perpetuating immune

responses (11-13). Alarmins comprise a multitude of molecules, many of which have yet to be described. However, there are several identified alarmin cytokines, which are known to differentially induce specific types of immune responses. For instance, both IL-1a and HGMB1 cytokines have been associated with promoting the generation of T_H1 immune responses (14,15). Specific to this paper, Interleukin 33 (IL-33), a relatively new member of the IL-1 pro-inflammatory cytokine family, has been associated with T_H2-driven responses (16,17). Although earlier studies have shown that IL-33 plays a role in T_H2 immune responses, new confounding evidence also characterizes IL-33 as a potent T_H1-polarizing alarmin (18-21). Current studies show that IL-33 can contribute to the development of T_H1-like CD4 and CD8 T cell immunity against infectious diseases and cancer (20,21). We, ourselves, recently demonstrated that IL-33 could serve as an immunostimulatory molecule to generate effective immune responses by inducing potent CD4 T_H1 and CD8 antiviral and antitumor immunity in a DNA HPV16 vaccine setting (21). Moreover, a recent study by Lee *et al.* reported a positive correlation between IL-33 and IFN γ levels in patients with TB pleurisy (22). Here, we investigated whether administration of IL-33 expressed as a DNA vaccine construct could increase the immunogenicity of an anti-TB DNA vaccine. We found that the co-immunization of IL-33 with a TB antigen 85B (Ag85B) DNA vaccine enhanced the potency of the Ag85B-specific CD4 T_H1 and CD8 T cell responses. This data encourages further studies investigating the potential use of IL-33 as an effective immunoadjuvant for future TB DNA vaccine studies and for protection in preclinical challenge models.

METHODS

DNA Construct Designs

The DNA constructs encoding mature IL-33 (mtrIL-33) and the TB Ag85B construct was designed as previously described (21,23). All constructs had highly efficient immunoglobulin E (IgE) leader sequences inserted at the 5' end of the gene. The constructs were commercially synthesized and optimized as described previously (21,23).

Animals

All animals were conducted and maintained in accordance with the NIH and the University of Pennsylvania Institutional Animal Care and Use Committee guidelines. Female C57BL/6 8-week-old mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Animal Immunizations

C57BL/6 mice (n = 4 per group) were immunized twice, two weeks apart, intramuscularly (i.m.) in the tibialis anterior muscle. *In vivo* electroporation (EP) was delivered, with the CELLECTRA adaptive constant current EP device (Inovio Pharmaceuticals), at the same site immediately following vaccination as previously described (21,23). The mice were immunized with either 10 µg pVAX1 or 10 µg Ag85B with or without 11 µg of mtrIL-33 construct. One week after final immunization, mice

were sacrificed and splenocytes were harvested to measure immune responses. All studies were repeated at least twice.

ELISPOT Assay

All spleens were processed and IFN γ ELISpot assays were performed to determine antigen-specific cytokine secretion. Briefly, spleens were collected in RPMI 1640 medium (supplemented with 10% FBS, 1X Antibiotic-Antimycotic, and 1X β -ME) and splenocytes were isolated by mechanical disruption of the spleen using a Stomacher machine (Seward Laboratory Systems, Bohemia, NY). The resulting mashed spleens were filtered using a 40 μ m cell strainer, treated with ACK lysis buffer for 5 minutes to lyse RBCs, washed in PBS and then re-suspended in RPMI medium for use in ELISpot or Flow Cytometry assay. The IFN γ ELISPOT assays were conducted as previously described in detail (21,23). Ag85B-specific T cell responses were measured by stimulating splenocytes with pooled peptides (11-mers overlapping by 8 amino acids; 2.5 μ g/ml final) spanning the entire TB Ag85B antigen (Invitrogen). All peptides were synthesized from GenScript. Concavalin A (Sigma-Aldrich, St. Louis, MO) was used as positive control and complete culture medium was used as negative control. Background staining from cells stimulated with medium alone has been subtracted. Spots were enumerated using an automated ELISPOT reader (Cellular Technology, Shaker Heights, OH).

Intracellular Cytokine Staining Flow Cytometry

Intracellular cytokine staining was performed after 5 hours of *ex vivo* stimulation with Ag85B pooled peptides as described (21-24). In cultures being used to measure degranulation, anti-CD107a (FITC; clone 1D4B; Biolegend) was added during time of stimulation to capture the degranulation induced by exposure to antigen stimulation by Ag-specific cells. The cells were then fixed and stained as described elsewhere (21-24). The following antibodies were used for surface staining: LIVE/DEAD Fixable Violet Dead Cell stain kit (Invitrogen), CD4 (V500; clone RM4-5; BD Biosciences), CD8 (APC-Cy7; clone 53-6.7; BD Biosciences). For intracellular staining the following antibodies were used: IFN γ (APC; clone XMG1.2; Biolegend), TNF α (PE; clone MP6-XT22; ebioscience), CD3 (PerCP/Cy5.5; clone 145-2C11; Biolegend), and IL-2 (PeCy7; clone JES6-SH4; ebioscience). All data was collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR) with SPICE v5.3 (free available from <http://exon.niaid.nih.gov/spice/>). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals. For flow cytometry, cells were gated on singlets using SSC-H by SSC-A followed by gating on LIVE-DEAD, CD3⁺CD4⁺CD8⁻ T and CD3⁺CD8⁺CD4⁻ T cells to examine the CD4 and CD8 T-cell populations.

Statistical Analysis

Group analyses were completed by matched, two-tailed, unpaired student's *t*-tests to analyze statistical significance of all quantitative data produced in this study. A *P*<0.05 was considered statistically significant. All values are mean \pm SEM and statistical analyses were performed using GraphPad Prism (La Jolla, CA).

RESULTS

IL-33 enhances TB Ag85B-specific IFN γ responses after *in vivo* vaccination

We have previously shown that IL-33 delivered as an immunoadjuvant can induce T_H1 responses in a DNA vaccine setting (21). Therefore we examined whether IL-33 could increase vaccine potency when co-administered with a DNA vaccine encoding the TB antigen 85B (Ag85B). To this end, C57BL/6 mice were immunized intramuscularly with 10 μ g of Ag85B with or without 11 μ g of IL-33, immediately followed by electroporation (EP) (**Figure 4.1A**). One week after final immunization the magnitude of Ag85B-specific IFN γ production was measured by IFN γ ELISpot assay. As shown in **figure 4.1B**, the addition of IL-33 increased the Ag85B-specific IFN γ T cell-secreting responses. Compared to the Ag85B alone-immunized group (~333 SFC per million splenocytes), the IL-33 vaccinated group resulted in a 3-fold increase (~1062 SFC per million splenocytes) in the frequency of Ag85B-specific responses (**Figure 4.1B**). These results suggest that IL-33 functions as an effective adjuvant to augment the TB antigen-specific responses during DNA vaccination.

IL-33 significantly increases TB Ag85B-specific CD4 and CD8 splenic T cell responses

Next we were interested in whether IL-33 could enhance both the CD4 and CD8 T cell responses to Ag85B, so we carefully characterized the phenotype and cytokine profile of the 85B-specific T cells generated. To that end, one week after last immunization (**Figure 4.1A**), splenocytes were stimulated *ex vivo* with Ag85B pooled

peptides and the production of IFN γ , TNF α , and IL-2 by CD4 and CD8 T cells was analyzed by intracellular cytokine staining (ICS). Compared to Ag85B vaccination alone, the IL-33 adjuvant group induced significantly higher percentages of Ag85B-specific CD4 T cells, many of which were multifunctional (**Figure 4.2D**). These T cells were described by total IFN γ (Ag85B: 0.8%; mtrIL-33: 1.6%), TNF α (Ag85B: 0.9%; mtrIL-33: 1.6%), and IL-2 cytokine production (Ag85B: 0.30%; mtrIL-33: 0.51%) (**Figures 4.2A-C**). As shown in **figure 4.2D**, vaccination with Ag85B co-administered with IL-33 elicited substantially higher frequencies of TB-specific CD4 T cells producing dual IFN γ ⁺TNF α ⁺ (Ag85B: 0.43%; mtrIL-33: 0.95%) or triple-positive IFN γ ⁺TNF α ⁺IL-2⁺ (Ag85B: 0.24%; mtrIL-33: 0.47%) in the spleens (**Figure 4.2D**). In terms of CD8 T cells, we saw a similar trend with the vaccinated IL-33 group, which induced higher percentages of TB-specific CD8 T cells producing total IFN γ (Ag85B: 0.19%; mtrIL-33: 1.12%), total TNF α (Ag85B: 0.29%; mtrIL-33: 1.1%), and total IL-2 cytokine responses, although IL-2 responses were not significant (**Figures 4.3A-C**). However, we found that immunization with IL-33 predominately elicited higher frequencies of TB-specific CD8 T cells producing dual IFN γ ⁺TNF α ⁺ (Ag85B: 0.12%; mtrIL-33: 0.92%)(**Figure 4.3D**). The administration of IL-33 produced similar amounts of Ag-specific CD4 and CD8 T cell responses with the majority of T cell subsets simultaneously secreted both IFN γ and TNF α . Subsequently, we analyzed the cytotoxic ability of the vaccine-induced CD8 T cells to undergo degranulation, which was measured by staining with antibody to CD107a, a marker for degranulation. Compared to Ag85B alone-vaccinated mice (IFN γ ⁺CD107a⁺: 0.2%), the CD8 T cells isolated from mice vaccinated with IL-33 showed significantly higher percentages of Ag-specific CD8⁺CD107a⁺ T cells secreting IFN γ (IFN γ ⁺CD107a⁺: 1%) (**Figure 4.4A**). The IL-33 immunized mice also elicited substantially higher polyfunctional

CD8⁺ T cells co-expressing CD107⁺IFN γ ⁺TNF α ⁺ (Ag85B: 0.11%; mtrIL-33: 0.91%), compared with the control groups (**Figure 4.4B**). Altogether, the inclusion of IL-33 can elicit robust levels of TB-specific T_H1 driven cell-mediated immune responses.

FIGURES

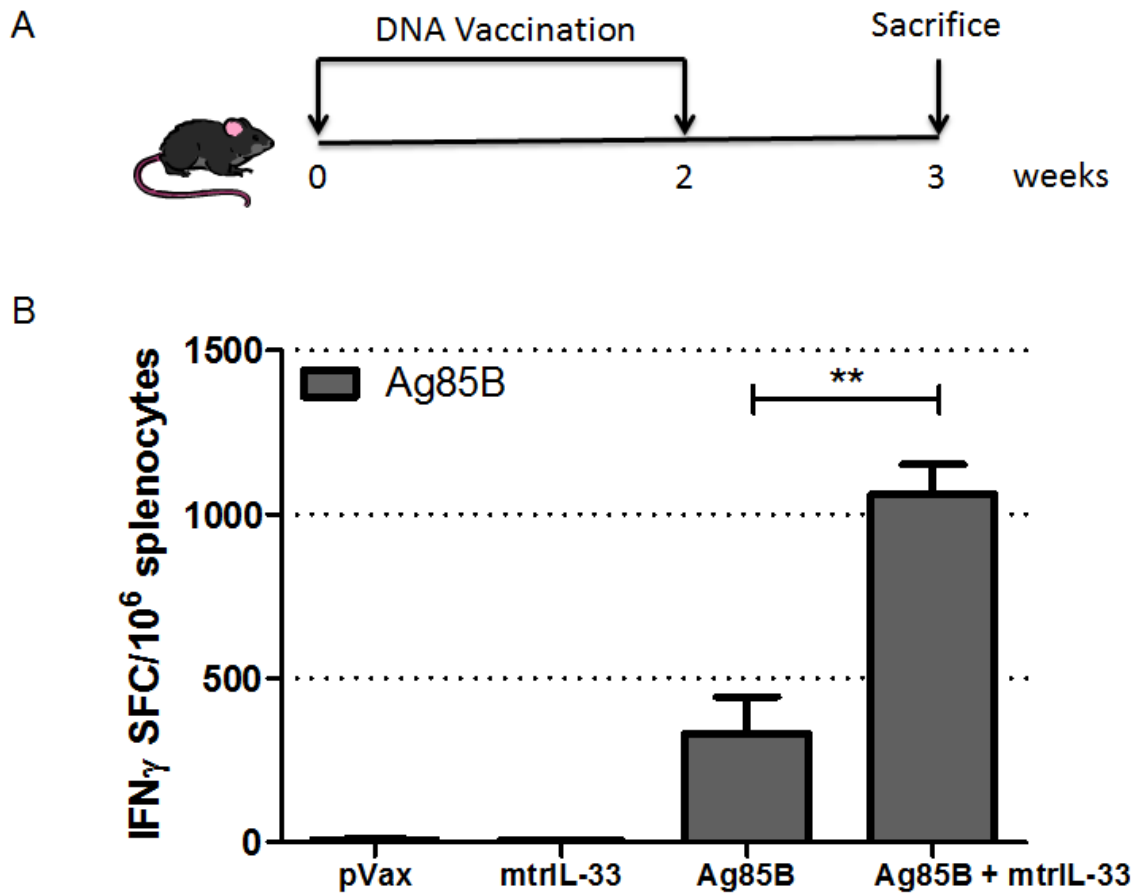


Figure 4.1. IL-33 enhances TB-specific IFN γ cellular immune responses in immunized mice. (A) Immunization schedule in mice. C57BL/6 mice ($n = 4$) were immunized twice, with a two-week interval between immunizations, with 10 μ g Ag85B construct with or without 11 μ g of IL-33 construct. (B) The total magnitude of IFN γ responses induced by isolated mice splenocytes ($n = 4$) stimulated ex vivo with Ag85B pooled peptides for 24 hours and measured by IFN γ ELISpot assay. The Data shows the SEM of one experiment repeated at least two times. **, $P < 0.01$

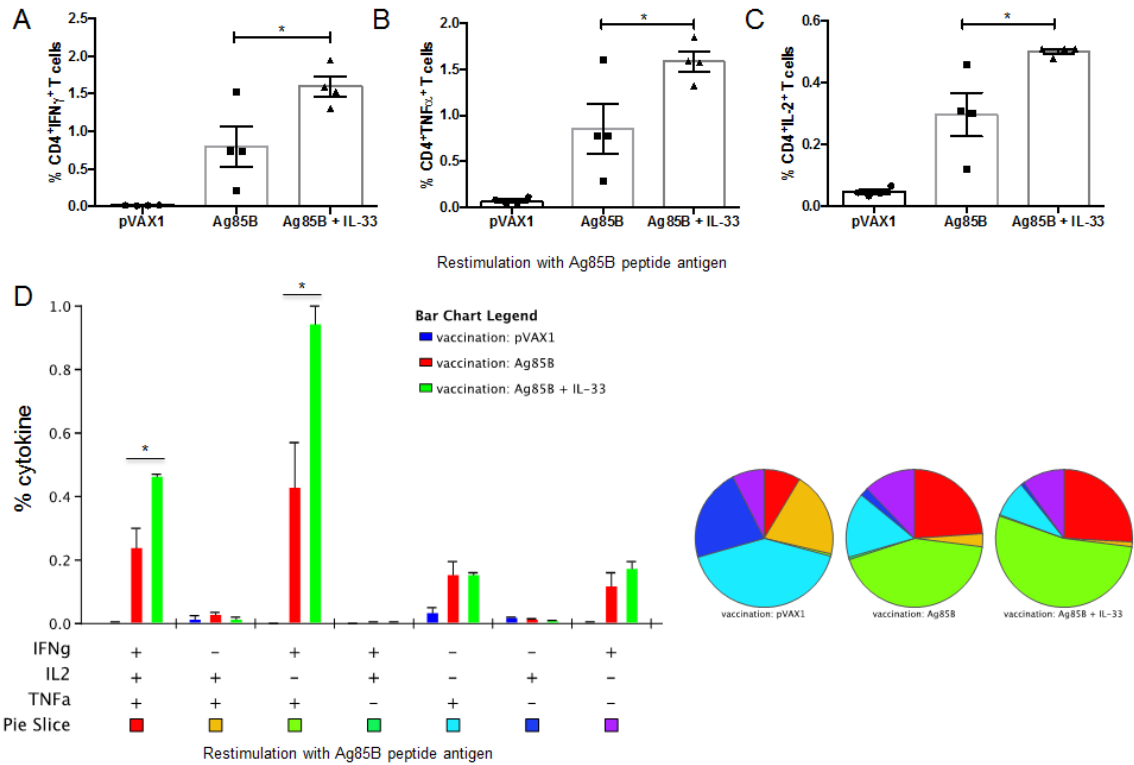


Figure 4.2. IL-33 augments cytokine production by Ag85B-specific CD4 T cells following DNA immunization. Cytokine-recall responses to TB Ag85B antigen were measured one week after last immunization by ICS and flow cytometry. A-C, column graphs depict the total TB-specific CD4 T cells expressing IFN γ (A), TNF α (B) and IL-2 (C). (D) Polyfunctional flow cytometry was used to determine the percentages of multifunctional CD4 T cell cytokine profiles. The bar chart shows the percentage of Ag85B-specific CD3⁺CD4⁺ T cells displaying triple, double, or single release of the cytokines IFN γ , TNF α , and/or IL-2⁺. Pie charts show the proportion of each cytokine subpopulation to Ag-specific stimulation. Experiments were performed independently at least twice and data represent the mean \pm SEM of four mice per group. *, P < 0.05 compared with Ag85B non-adjuvanted group.

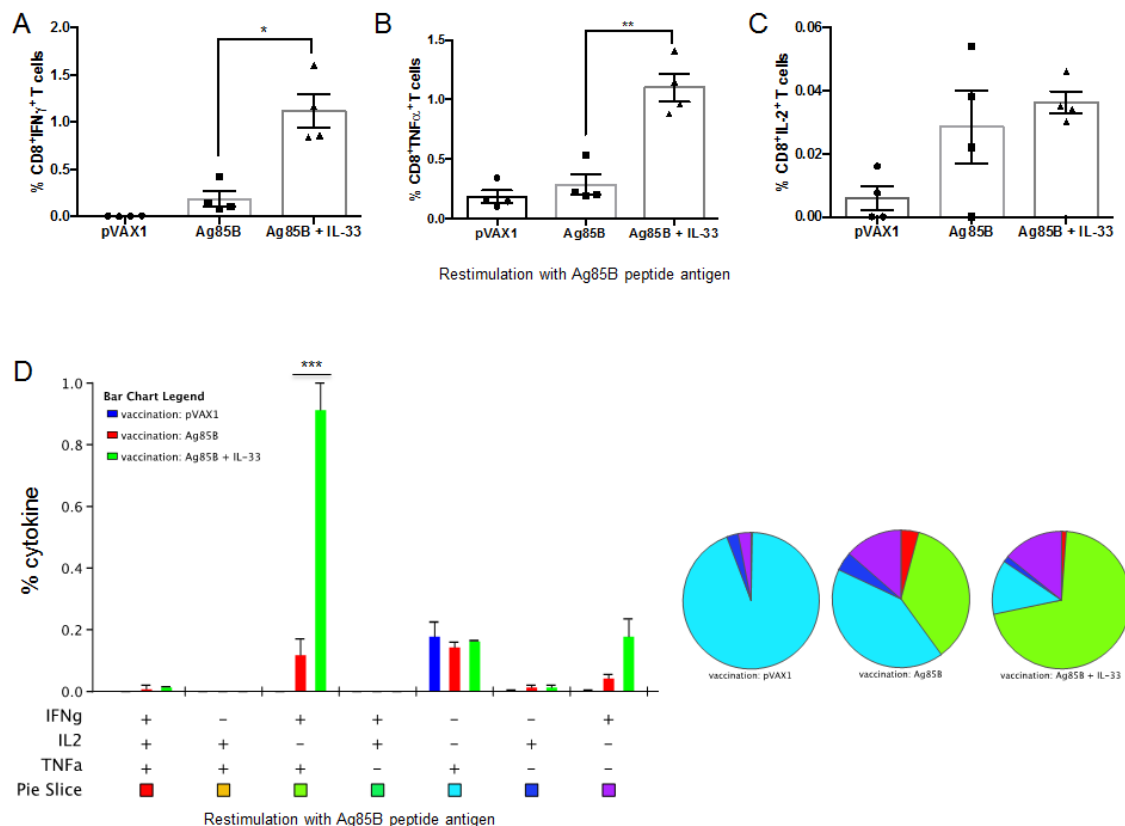


Figure 4.3. Induction of enhanced cytokine production of Ag85B-specific CD8 T cells following DNA immunization with IL-33. Cytokine-recall responses to TB Ag85B antigen were measured one week after last immunization by ICS and flow cytometry. A-C, column graphs depict the total TB-specific CD8 T cells expressing total IFN γ (A), TNF α (B) and IL-2 (C). (D) Polyfunctional flow cytometry was used to determine the percentages of multifunctional CD8 T cell cytokine profiles. The bar chart shows the percentage of Ag85B-specific CD3⁺CD8⁺ T cells displaying triple, double, or single release of the cytokines IFN γ , TNF α , and/or IL-2. Pie charts show the proportion of each cytokine subpopulation to Ag-specific stimulation. Experiments were performed independently at least twice and data represent the mean \pm SEM of four mice per group. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ compared with Ag85B non-adjuvanted group.

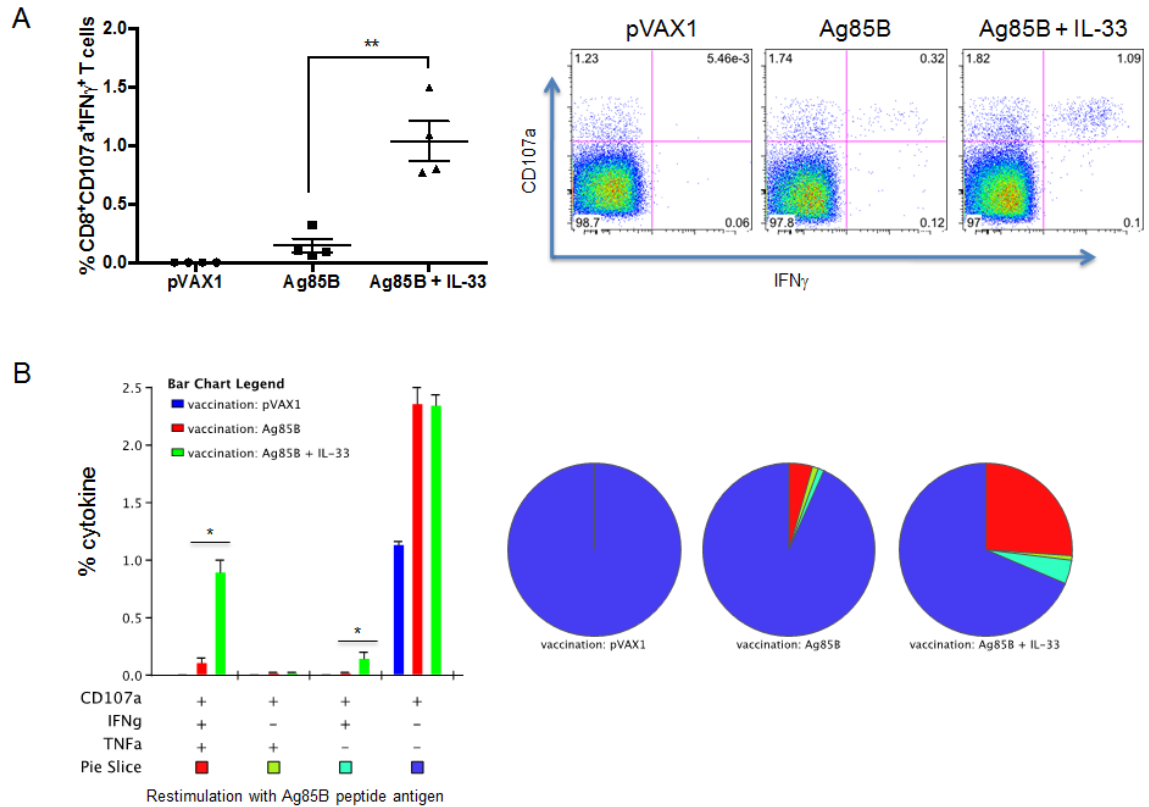


Figure 4.4. IL-33 promotes Ag85B-specific cytotoxic degranulating CD8 T co-expressing IFN γ and TNF α . (A) Ag85B-specific, cytolytic-degranulation CD8 T cells were measured by degranulation marker expression, CD107a and IFN γ . Figure (B) shows the frequency of polyfunctional CD8 T cells co-expressing CD107a. Data represent the SEM of 4 mice per group. The experiment was repeated twice with similar outcome. **P < 0.01, *P < 0.05 using Student's t-test.

DISCUSSION

It has been established that the development of an acquired cellular immune response (both T_H1 -type CD4 and CD8 T cells) is paramount for the control of Mtb infection. These T cell populations secrete essential cytokines ($IFN\gamma^+$ and $TNF\alpha^+$), which stimulate infected macrophages to kill intracellular bacteria (25-27). Additionally it has been shown that knockout mice that have been genetically altered to eliminate $IFN\gamma^+$ or $TNF\alpha^+$ production are more susceptible to mycobacterial infection (25-27). Therefore, the development of new molecular adjuvants to drive these preferable anti-TB immune responses may potentially lead to a more effective vaccine against TB.

The data presented here demonstrates that IL-33 delivered as a molecular adjuvant can evoke significant Ag85B-specific cell-mediated immune responses in a TB DNA vaccine setting. We show that co-administration of IL-33 with Ag85B DNA EP immunization markedly increases the magnitude of Ag-specific $IFN\gamma$ responses by ELISpot. The inclusion of IL-33 induced a ~3-fold greater response compared with to Ag85B-alone immunization (**Figure 4.1B**). We find this in accordance with previous data showing that administration of IL-33 along with vaccine antigen can increase the vaccine-induced T_H1 responses (20,21). Furthermore, we show that IL-33 can increase the total splenic CD4 T cells and CD8 T cells secreting $IFN\gamma$ and $TNF\alpha$ after Ag85B peptide pool stimulation (**Figures 4.2-4.3**). Given the importance of multifunctional vaccine-induced anti-TB protective CD4 T cell responses to prevent disease after exposure and to control Mtb in a latent state (28,29), we assessed the polyfunctionality of the CD4 T cells (**Figure 4.2**). The Ag85B vaccine co-administered with IL-33 elicited a significant enhancement of the polyfunctional CD4 T cell population simultaneously secreting both $IFN\gamma^+TNF\alpha^+$ and $IFN\gamma^+TNF\alpha^+IL-2^+$ cytokines (**Figure 4.2D**). The triple

positive $\text{IFN}\gamma^+\text{TNF}\alpha^+\text{IL-2}^+$ and double positive $\text{IFN}\gamma^+\text{TNF}\alpha^+$ T cell phenotypes normally represent effector memory and central memory T cells (30), indicating the induction of memory CD4 T cell immune responses. Within the cytokine-producing CD8 T cells, the proportional distribution of polyfunctional T cell subsets followed the order of $\text{IFN}\gamma^+\text{TNF}\alpha^+$ being greater than $\text{IFN}\gamma^+$ (**Figure 4.3D**). IL-33 not only improved the polyfunctionality of the CD8 T cells, but also increased their antigen-specific cytolytic phenotype activity as demonstrated by the co-expression of $\text{CD107a}^+\text{IFN}\gamma^+\text{TNF}\alpha^+$ (**Figure 4.4B**). Moreover, IL-33 significantly enhanced the vaccine-induced total CD4 T cells secreting IL-2 responses (**Figure 4.2C**), a cytokine that is secreted predominately by Ag-stimulated CD4^+ T cells (31-33). IL-2 also plays a crucial role in driving CD8 T cell differentiation, proliferation, and activation and therefore enhancing the Ag-specific CD8 effector functions (**Figure 4.3 & 4.4**), such as cytolytic activity and cytokine production (8,9). Altogether, the data highlight adjuvant effects of IL-33 to augment vaccine potency of both CD4 and CD8 T cells, which would be indispensable for protective immunity against TB (8-10).

These results are in agreement with our previously published data that demonstrated that IL-33 could enhance both CD4 $\text{T}_\text{H}1$ and CD8 T cell responses with cytolytic properties (21). Furthermore, this finding is consistent with the recent reports that IL-33's role extends beyond $\text{T}_\text{H}2$ immunity; IL-33 can promote $\text{T}_\text{H}1$ and CD8 type cell-mediated immunity given the appropriate cytokine milieu and microenvironment (16-21). Recent reports suggest that IL-33 can be a potent inducer of CD8^+ T cells, as only activated effector CD8 T cells can up-regulate the IL-33 cognate receptor, ST2, and in synergy with IL-12 can selectively enhance the expression of $\text{IFN}\gamma$ responses (20). The mechanism by which IL-33 promotes $\text{T}_\text{H}1$ CD4 and CD8 T cell differentiation is still unknown. In our study, it is possible that the co-induction of type 1 cytokine $\text{IFN}\gamma$ could

be related to our selected antigen, Ag85B, which is a potent T_H1 antigen (34,35). Therefore, the favored T_H1 cytokine milieu perpetuated by our selected TB antigen may have induced a favorable immune environment that allowed IL-33 to foster a greater T_H1 cell-mediated immune response. Further studies are needed to understand exactly how IL-33 induces type-1 T_H1 IFN γ ⁺ responses in the context of an *in vivo* immune setting. In addition, studies are needed to understand how IL-33 bridges the innate with the adaptive immune response to evoke a pro-T_H1 cell-mediated response.

Overall, in this study we further validate IL-33 as a future potent T_H1- and CD8-mediated immunoadjuvant in a DNA vaccine setting. IL-33 may be an effective strategy for increasing the efficacy of future DNA vaccines against Mtb. Since Mtb infection targets the lungs, current studies are evaluating the ability of IL-33 to induce polyfunctional antigen-specific T cells in the lungs. Furthermore, although IL-33 can elicit desired cell-mediated responses, it can also be detrimental to subjects at risk for HIV. The immune activation induced by IL-33 could pave the road for more efficient acquisition of HIV infection. Thus, challenge studies will be important to confirm the protective nature of IL-33 as a vaccine adjuvant in a relevant challenge system. Experiments are currently under way to test the protective efficacy of IL-33 against Mtb both as a standalone and as a prime-boost immunization regimen in combination with BCG.

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CHAPTER 5

UBIQUITIN-LIKE MOLECULE ISG15 ACTS AS AN IMMUNE ADJUVANT TO ENHANCE ANTIGEN-SPECIFIC CD8 T CELL TUMOR IMMUNITY

*"This day is full of glorious victory,
Echoes of conquest whisper from afar
In every wave of the remembering sea."*

-H. Begbie

Abstract

ISG15 is an ubiquitin-like protein induced by type I interferon associated with antiviral activity. ISG15 is also secreted and known to function as an immunomodulatory molecule. However, ISG15's role in influencing the adaptive CD8 T cell responses has not been studied. Here, we demonstrate the efficacy of ISG15 as a vaccine adjuvant, inducing HPV E7-specific IFN γ responses as well as the percentage of effector-like memory, polyfunctional, and cytolytic CD8 T cell responses. Vaccination with ISG15 conferred remarkable control and/or regression of established HPV-associated tumor-bearing mice. T cell depletion coupled with adoptive transfer experiments revealed that ISG15 protective efficacy was CD8 T cell-mediated. Importantly, we demonstrate that ISG15 vaccine-induced responses could be generated independent of ISGylation, suggesting that responses were mostly influenced by free ISG15. Our results provide more insight into the immunomodulatory properties of ISG15 and its potential to serve as an effective immune adjuvant in a therapeutic tumor or infectious disease setting.

Introduction

The induction of cytotoxic CD8 T cells is believed to be essential in tumor control, and, thus, a necessary goal for any therapeutic cancer vaccine. Nevertheless, insufficient generation of CD8 effector T cells has led to the failure of several therapeutic cancer vaccines to produce clinical regression of solid tumors (1-3). For such vaccines, the incorporation of adjuvants can assist in generating potent and durable tumor immunity (4,5), but most of the effects of adjuvants have been limited to T_H1 CD4 expansion with poor CD8 T cell killing function induced. Thus identifying adjuvants capable of amplifying CD8 T cell antitumor immunity is very important for therapeutic antitumor vaccines.

Interferon-stimulating gene 15 (ISG15) is one of the first and most abundant proteins induced by type I interferon stimulation (6). ISG15 is an ubiquitin-like protein, which plays a major role in antiviral defense (6). Its ubiquitin-like C-terminal (LRLRGG) motif is necessary for its conjugation to a variety of intracellular proteins in a process known as ISGylation (6) producing “conjugated” ISG15. When not in its conjugated form, free or “unconjugated” ISG15 can exist intracellularly or extracellularly. For decades, free ISG15 has been implicated in the production of IFN γ (7-9). Recently, a new study confirmed this cytokine-like role for ISG15 by demonstrating that ISG15- deficiency was associated with a loss of IFN γ , which in turn led to increased susceptibility to mycobacterial disease in both mice and humans (10). Although these studies have established the ability of ISG15 to function as an immunomodulatory molecule, its ability to influence CD8 T cell immune responses and act as a vaccine adjuvant remains unknown. Here we sought to investigate the role of ISG15 as an adjuvant to enhance tumor-specific CD8 T cell immunity using a human papilloma virus (HPV)-associated tumor murine therapeutic model.

Here we report that ISG15 can act as an effective CD8 T cell-mediated adjuvant when co-delivered with a HPV16 DNA vaccine via *in vivo* electroporation (EP). The inclusion of ISG15 substantially increased E7-specific IFN γ responses as well as the percentage of effector-like memory, polyfunctional, and cytolytic CD8 T cells. Importantly, we report that the augmentation of ISG15's functional CD8-mediated tumor immunity achieved control and/or regression of tumors in established HPV-associated tumor-bearing mice. We also show that the therapeutic efficacy of ISG15 correlates with the increase in magnitude and phenotype of tetramer-specific, effector-memory CD8 T cells. Finally, we demonstrate that ISG15 delivered as an immunoadjuvant generates responses independent of conjugation as an LRLRGG-mutant ISG15 also induced potent CD8 T cell responses. We conclude that ISG15 may be a valuable tool to improve the immunogenicity of vaccines against cancer as well as to treat persistent infections.

METHODS

DNA construction and expression

The GenBank accession no. Q64339 for mouse ISG15 was used to synthesize the DNA construct encoding wild-type ISG15 (wtISG15). Mutated ISG15 (mutISG15) is a variant of wtISG15 with point mutations at its C-terminal conjugation site (LRLRGG to AAAAGG). All constructs contained highly efficient immunoglobulin E (IgE) leader sequence inserted at the 5' end of the gene. The constructs were commercially synthesized and optimized as described previously (5,11). HPV16 plasmid containing the E6 and E7 antigens was prepared as previously described (15). *In vitro* expression of both ISG15 constructs was confirmed by Western Blot (WB) analysis using. Human rhabdomyosarcoma (RD) cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island NY USA) and supplemented with 10% heat-inactivated fetal calf serum as well as penicillin and streptomycin. After plating 3.0×10^5 cells per well, transfection was performed using Neofectin (NeoBiolab Cambridge MA) following the manufacture's protocol. Cells were transfected with 2 μ g of each DNA construct including pVAX1 empty vector backbone as a negative control. Following 48 hour incubation, cell supernatants were collected and cells were washed with cold PBS. After centrifugation, cells were lysed using cell lysing buffer (Cell Signaling Technology Danvers, MA) and EDTA free protease inhibitor cocktail (Sigma-Aldrich St. Louis, MO). Cell lysate was run on a 10% Tris-Acetate gel with MES buffer (Life Technologies Grand Island NY USA) and transferred onto a PVDF membrane (Millipore, Darmstadt, Germany). The membrane was block using Odyssey blocking buffer (Licor, Lincoln, Nebraska) for three hours at room temperature followed by probing with rabbit anti-mouse ISG15 (Cell Signaling Technology Danvers, MA) and mouse anti-human β -actin (Sigma-Aldrich St. Louis, MO) as a loading control at 4 degrees overnight. After washing

with PBS-Tween, secondary goat anti-mouse IRDye 680RD and goat anti-rabbit IRDye 800 CW (Li-cor, Lincoln, Nebraska) were added for 1 hour at room temperature. The membrane was then washed and imaged on the Odyssey CLX (Licor, Lincoln, Nebraska). In addition, supernatants were also collected at 48 hours after transfection and cytokine secretion was examined by using a CircuLex mouse ISG15 ELISA kit (MBL International), according to manufacturer's protocol. Optical density was measured at 450nm using a Biotek EL312e Bio-Kinetics reader (Biotek US, Winooski, VT). All supernatants were tested in duplicate with two separate supernatant sample per a plasmid.

Animals

All animals were conducted and maintained in accordance with the NIH and the University of Pennsylvania Institutional Animal Care and Use Committee guidelines. Female C57BL/6 (H-2^b) 8-week-old mice and H2^b B6.129S7-Rag1^{tm1Mom}/J mice (Rag1 KO) were purchased from Jackson Laboratory.

Animal immunizations

All mice were immunized intramuscularly (i.m.) in the tibialis anterior muscle. *In vivo* electroporation (EP) was delivered, with the CELLECTRA adaptive constant current electroporation device (Inovio Pharmaceuticals), at the same site immediately following immunization as previously described (11). The mice were immunized with either 5µg pVAX1 or 5µg of HPV16 construct with or without 11µg of wtISG15 and mutISG15. All studies were performed at least twice.

ELISPOT assay

Spleens were harvested and processed 7 days following the final immunization as previously described (5,11). After spleens were harvested and processed, an IFN γ ELISpot assay was performed to determine antigen-specific cytokine secretion from immunized mice as described previously in detail (5,11-12). HPV16 Ag-specific T cell responses were measured by stimulating splenocytes with E6 or E7 pooled overlapping peptides (2.5 μ g/ml final concentration of peptide). The E7 overlapping pooled peptides contained the CD8 T cell immunodominant HPV16 D^bE7₄₉₋₅₇ epitope (RAHYNIVTF).

Flow cytometry

Lymphocytes were isolated and processed from the spleen and peripheral blood as previously described (5,11,13). Lymphocytes were stained with CD8, KLRG1, and MHC class I peptide tetramer to HPV16 H-2D^bE7₄₉₋₅₇ (RAHYNIVTF) (MBL International) as described previously (5,14). Intracellular cytokine staining was performed after 5 hrs of *ex vivo* stimulation with the HPV16 E7 peptide D^bE7 (RAHYNIVTF) (2.5 μ g/ml final concentration of peptide) or E7 pooled overlapping peptides to assess CD4 T responses (12). In cultures being used to measure degranulation, anti-CD107a (FITC; clone 1D4B; Biolegend) was added during time of stimulation to capture the degranulation induced by exposure to stimulation by Ag-specific cells (5). The cells were then fixed and stained as described elsewhere (5,15). The following antibodies were used for surface staining: LIVE/DEAD Fixable Violet Dead Cell stain kit (Invitrogen), CD4 (FITC; clone RM4-5; ebioscience), CD8 (APC-Cy7; clone 53-6.7; BD Biosciences), NK1.1 (FITC; clone PK136; biolegend); CD49b (FITC; clone DX5; ebioscience). For intracellular staining the following antibodies were used: IFN γ (APC; clone XMG1.2; Biolegend), TNF α (PE; clone

MP6-XT22; ebioscience), CD3 (PerCP/Cy5.5; clone 145-2C11; Biolegend); IL-2 (PeCy7; clone JES6-SH4; ebioscience). All data was collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR) and SPICE v5.3 (free available from <http://exon.niaid.nih.gov/spice/>). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals.

Tumor cell line

The TC-1 cell line was a graciously given gift from Dr. Yvonne Paterson of the University of Pennsylvania, Philadelphia, PA. TC-1 cell line is a well-characterized lung epithelial immortalized cell line, constitutively expresses E6 and E7, and is highly tumorigenic (16,17). The TC-1 cells were purchased from American Type Culture Collection and cultured as previously described (5).

***In vivo* therapeutic study**

B6 mice were separated into four groups of 10 mice each and 5×10^4 TC-1 cells were subcutaneously implanted into the right flank of each mouse. On day 4, after tumor implantation, each group of mice was immunized by intramuscular electroporation with pVAX1, HPV16, HPV16/wtISG15 or HPV16/mutISG15 and boosted on days 11, 18, and 25. Tumor size was measured twice a week using electronic calipers and tumor volume calculated as described previously [$\frac{1}{2}(\text{length} \times \text{width}^2)$]. Mice were monitored twice a week for tumor growth and were measured as described previously (5,12). Under Penn Institutional Animal Care guidelines, mice were sacrificed when tumor size reached 18-20mm.

***In vivo* CD8 T cell depletion study**

During therapeutic vaccination, B6 mice were injected intraperitoneal with 200 µg of anti-CD8 (53-6.72, Bio X cell) one day before tumor inoculation and repeated every three days thereafter. Successful T cell depletion was confirmed by flow cytometric analysis of peripheral blood mononuclear cells.

T-cell purification and adoptive transfer

CD8 T cells were isolated from splenocytes of vaccinated B6 mice 1 week after final immunization in non-bearing tumor mice (Fig 2A). CD8⁺ T cells were purified from splenocytes using negative selection to deplete CD4⁺ T cells, B cells, and myeloid cells. Briefly, following RBC lysis, splenocytes were incubated with rat IgG anti-CD4 (GK1.5), anti-B220 (RA3), anti-CD11b (M170.13), anti-MHC-II (M5/114), and anti-CD16/32 (2.4G2). Antibody-bound cells were removed using anti-rat IgG magnetic beads (18). For adoptive transfer, $\sim 4 \times 10^6$ CD8 T cells in 200 µl PBS were injected intravenously via tail vein into each H2^b B6.129S7-Rag1^{tm1Mom}/J mouse.

Statistical Analysis

Group analyses were completed by matched, two-tailed, unpaired student's *t*-tests to analyze statistical significance of all quantitative data produced in this study. A P-value <0.05 was considered statistically significant. Error bars indicate SEM and all tests were performed using the Prism Software (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 compared with HPV16 immunization).

RESULTS

Design and expression of ISG15 constructs

The wild-type ISG15 (wtISG15) adjuvant construct was generated using the mouse ISG15 sequence retrieved from GenBank (accession number: Q64339) with several modifications (**Figure 5.1A**). ISG15 contains a C-terminal LRLRGG motif that is necessary for its conjugation to a variety of target proteins in a process referred to as ISGylation (19-21). In order to determine if conjugation was necessary for ISG15-mediated immunomodulation, the ISG15 conjugation sequence site was mutated (LRLRGG to AAAAGG) to generate the mutant ISG15 (mutISG15), incapable of conjugation (**Figure 5.1A**). Both ISG15 constructs were genetically optimized and subcloned into a modified pVAX1 mammalian expression vector (**Figure 5.1B**). To verify the expression of both ISG15 encoding constructs, human rhabdomyosarcoma (RD) cells were transfected separately with each vector and examined by WB. As shown in **Figure 5.1C**, an ~15kDa free ISG15 was observed for each in cell lysates harvested 48 hours after transfection using anti-ISG15 monoclonal antibody (mAb) for detection. ISG15 expression was not detected in the negative control pVAX1 group. Next, via an enzyme-linked immunosorbent assay (ELISA) the secretion of free ISG15 was monitored from the cell supernatants that were obtained 48 hours after transfection of RD cells. As projected, supernatants from mutISG15 transfected RD cells had a higher concentration of detectable secreted free ISG15 (7.2 ng/ml), compared to wtISG15 (4.4 ng/ml) (**Figure 5.1D**). This supported our notion that by mutating ISG15's conjugation motif, more unconjugated ISG15 would be available and secreted to the extracellular environment.

Immunization with ISG15 adjuvant induced strong HPV E7-specific CD8 T cell immune responses.

To assess the immunogenic properties of ISG15, an IFN γ ELISpot assay was performed to determine the number of vaccine-induced E7-specific IFN γ secreting cells in response to E7 pooled peptides containing the immunodominant CTL epitope H-2-D^bE7₄₉₋₅₇ (E7). The immunization regimen is shown in **Figure 5.2A**. Briefly, groups of B6 mice (n = 4-5/group) were vaccinated twice at two-week intervals as follows: (i) HPV16 DNA/EP; (ii) HPV16/wtISG15 DNA/EP; (iii) HPV16/mutISG15 DNA/EP; and (iv) pVAX1/EP. The co-administration of HPV16 with wtISG15 resulted in a 3.5-fold increase in E7-specific IFN γ responses (~230 SFC/million splenocytes) compared with HPV16 alone-immunized group (~66 SFC/million splenocytes). ISG15 is an ubiquitin-like protein that conjugates to target proteins and is critical for control of certain viral and bacterial infections (6). In addition to the conjugated form of ISG15, it is known, that ISG15 is present in an unconjugated form (free ISG15) and can also play an important role in immunomodulation or during infection (6). Thus, in the same experiment, we examined if vaccine-induced responses were independent of conjugation by immunizing mice with a mutated form of ISG15 lacking a functional C-terminal LRLRGG conjugating motif. Interestingly, similar to wtISG15, the mutISG15 vaccinated group demonstrated an increase (~4-fold) in total E7-specific cells compared with HPV16-only group, suggesting ISG15 can induce its effects independent of conjugation. We did not find relatively higher induced levels of E6-specific vaccine-induced responses (data not shown). Together, these data suggest the ubiquitin-like molecule ISG15 can act as an adjuvant to enhance and stimulate E7-specific T_H1-mediated CD8 T cell responses. Moreover, this data

demonstrated that the elevated antigen (Ag)-specific responses were most likely attributed to free ISG15.

ISG15-mediated augmentation of polyfunctional HPV E7-specific cell-mediated responses

Considering that CD8⁺ T cell immune responses are essential in prevention of tumorigenesis and elimination of tumors (1,22-25), we further examined the functional profile of E7-specific CD8 T cell populations from vaccinated mice to secrete IFN γ , TNF α and IL-2 in response to D^bE7₄₉₋₅₇ peptide stimulation. Our gating strategy for intracellular cytokine multiparametric flow cytometry analysis is shown in **Figure 5.3A**. One week after final vaccination all tested vaccination regimens induced detectable CD8 T cell responses producing all three effector cytokines (**Figure 5.3**). Compared to the antigen alone group, both ISG15 vaccine regimens induced significant E7-specific CD8 T cells producing total IFN γ (wtISG15, 0.68%; mutISG15, 0.92%) (**Figure 5.3B**) and total TNF α (wtISG15, 0.42%; mutISG15, 0.54%) (**Figure 5.3C**). However, ISG15 only induced a minor increase of Ag-specific CD8 T cells secreting IL-2 (**Figure 5.3D**). Importantly, a significant number of the E7-specific CD8 T cells were polyfunctional, with ISG15-immunized groups eliciting significantly higher frequencies of CD8 T cells producing either IFN γ alone or dual IFN γ ⁺TNF α ⁺ in the spleens 7 days post vaccinations (**Figure 5.3E**). There was also a modest increase in the triple-positive IFN γ ⁺TNF α ⁺IL-2⁺ CD8 secreting cells in the ISG15-treated groups. Since ISG15 can have an effect on NK cells, we monitored vaccine-induced NK responses, but no significant changes were seen after vaccination with ISG15 (**Figure 5.4A**) (9). Furthermore, the administration of ISG15 did not increase vaccine-induced CD4 T cell responses as measured by *ex vivo* E7

pooled peptide stimulation (**Figure 5.4B**).

Given that cytotoxic CD8 T lymphocytes (CTL) are critical components in protection (1,5,24,25), we assessed the cytolytic properties of the adjuvant-induced CTL responses to undergo degranulation and secrete effector cytokines simultaneously (**Figure 5.5**). The groups vaccinated with immunoadjuvant ISG15 showed higher percentages of the degranulation marker, CD107a (wtISG15, 2.4%; mutISG15, 3.1%), compared with HPV16-alone group (**Figure 5.5A**). More interestingly, the ISG15-adjuvanted vaccines elicited substantially higher frequencies of polyfunctional CTLs, with a substantial representation of cells showing one, two, and three immunological functions (**Figure 5.5B-C**). Notably, compared to HPV16 administered alone, the ISG15-treated groups showed significantly higher frequencies of CD8 T cells co-expressing CD107a⁺IFN γ ⁺TNF α ⁺ (wtISG15, 0.35%; mutISG15, 0.43%) (**Figure 5.5C**). Collectively, the high frequencies of effector cells secreting proinflammatory cytokines are indicative of the ISG15 cytokine-like properties and its adjuvant effects to enhance vaccine potency by driving potent functional effector CTL immunity. Overall, an important observation here was that a DNA plasmid expressing the mutISG15, incapable of conjugation, maintained the adjuvant effects displayed by wild-type form, suggesting that ISGylation is likely not required for immunomodulation of CD8 T cells.

ISG15 adjuvant amplifies robust Ag-specific effector-memory CD8 T cell responses

Since it has been demonstrated that magnitude and quality of E7-specific CD8 T cell responses correlates with the therapeutic efficacy of HPV vaccine against established tumors (24,26), we investigated the HPV tetramer-specific CD8 T responses

that may correlate with vaccine-induced HPV-associated tumor control. To this end, non-tumor-bearing B6 mice were immunized with the aforementioned vaccination formulations and schedule in **Figure 5.2A**. One week after final immunization, the magnitude and subset differentiation of Ag-specific CD8 T cell responses were examined using the CD8 epitope specificity of HPV16 E7₄₉₋₅₇ H2-D^b-RAHYNIVTF tetramer in the spleen and blood (**Figure 5.6**). Both wtISG15 and mutISG15 constructs were able to significantly increase the D^bE7 tetramer-specific CD8 T cell responses in the spleen compared to HPV16 group alone (**Figure 5.6A and B**). In addition, the delivery of both ISG15 plasmids also significantly amplified the number of D^bE7 tetramer-specific CD8 T cells in the peripheral blood, suggesting trafficking of tumor target-specific CTL's into the periphery (**Figure 5.6E**) (26). The frequency of E7-tetramer T cells in the blood within the wtISG15 and mutISG15 groups were 4 and 5.5-fold higher compared with the nonadjuvanted group, respectively. This data confirmed that immunoadjuvant ISG15 could amplify the Ag-specific CD8 T cells.

It has been suggested that effector-memory CD8 T cells are optimal subsets for protective immunity and may predict therapeutic efficacy against tumors (4,5,27,28). Effector memory T cells are the focus of cancer vaccines as they can initiate rapid effector function and migrate quickly to the infected- or tumor-site (1,29-31). In this study, we measured the D^bE7 MHC class I tetramer vaccine-induced effector/effector-memory CD8 T cell subset based on expression marker of KLRG1 (effector memory - T_{eff}) (5,14,27) (**Figure 5.6**). The administration of wtISG15 resulted in a ~3-fold increase in the percentages of T_{eff} cells in the spleen, compared with the HPV16-only vaccinated group (**Figure 5.6C and D**). Similarly, the inclusion of mutISG15 also significantly enhanced the T_{eff} responses in the spleen (**Figure 5.6D**). In addition, as shown in **Figure 5.6F**, the percentages of T_{eff} cells in the blood were significantly higher in the adjuvant

groups. These data suggest that immunoadjuvant ISG15 can enhance the magnitude and quality of E7-specific CD8 T cell responses.

ISG15 acted as an effective CD8 T cell immunoadjuvant inducing antitumor immunity

We next determined the therapeutic efficacy of ISG15 in a TC-1 tumor-bearing mice model. Naïve recipient B6 mice (n = 10/group) were first inoculated subcutaneously with TC-1 tumor (5×10^4) cells followed by HPV16, HPV16/wtISG15, HPV16/mutISG15 or pVAX1 vaccination four days after tumor implantation (tumors had reached an average size of 2 mm), followed with three boosts at 1-week intervals (**Figure 5.7A**). Tumors in mice immunized with the mixture of HPV16/wtISG15 grew significantly slower than HPV16 vaccinated group alone (**Figure 5.7B**). In contrast, pVAX1 control group failed to show any therapeutic effect with all mice dying by day 35. Interestingly, mice given the HPV16/mutISG15 had significantly better tumor control than mice given HPV16/wtISG15, likely due to greater induction of tumor-specific CTL responses. In addition, compared to HPV16/wtISG15, the HPV16/mutISG15 combination rapidly induced regression of more established TC-1 tumors (**Figure 5.8**). At day 42 post tumor implantation, 6/10 mice in the HPV16-mutISG15 were tumor free, compared with either HPV16 (1/10) or HPV16-wtISG15 (2/10) (**Figure 5.8**). Taken together, the adjuvant properties of ISG15 demonstrated effective control and therapeutic cure of HPV-associated tumor-bearing mice.

Given ISG15 adjuvant properties to enhance E7-specific-CTL responses that are essential to target established preexisting HPV infections (22,23), we investigated the role of ISG15-elicited CD8 T cells for HPV-associated tumor elimination. Therefore, in a therapeutic setting, CD8 T cells were depleted by intraperitoneal injection of anti-CD8

antibody, beginning 1 day post-tumor inoculation and repeated every three days subsequently (**Figure 5.7C**). Our results revealed that CD8 depletion significantly abrogates the therapeutic effects of ISG15 adjuvancy as no mice survived to 30 days post-implantation (**Figure 5.7D**). To confirm these findings, we performed CD8 T cell adoptive transfer experiments in T cell immunodeficient B6 Rag1 KO mice (32). 4×10^6 CD8 T cells purified from splenocytes of HPV16, HPV16/wtISG15, and HPV16/mutISG15 immunized mice (**Figure 5.2A**) were injected intravenously 4 days post-inoculation of TC-1 cells (**Figure 5.7E**). As compared to HPV16 and naive controls, mice that received either wtISG15 or mutISG15 vaccine-induced CD8 T cells had significantly slower tumor growth (**Figure 5.7F**), likely owing to their functional CTL phenotype (**Figure 5.3** and **5.5**). Taken together, the results suggest that ISG15-elicited CD8 T cells are essential to prolonging survival and controlling tumor growth in the TC-1 therapeutic tumor model.

FIGURES

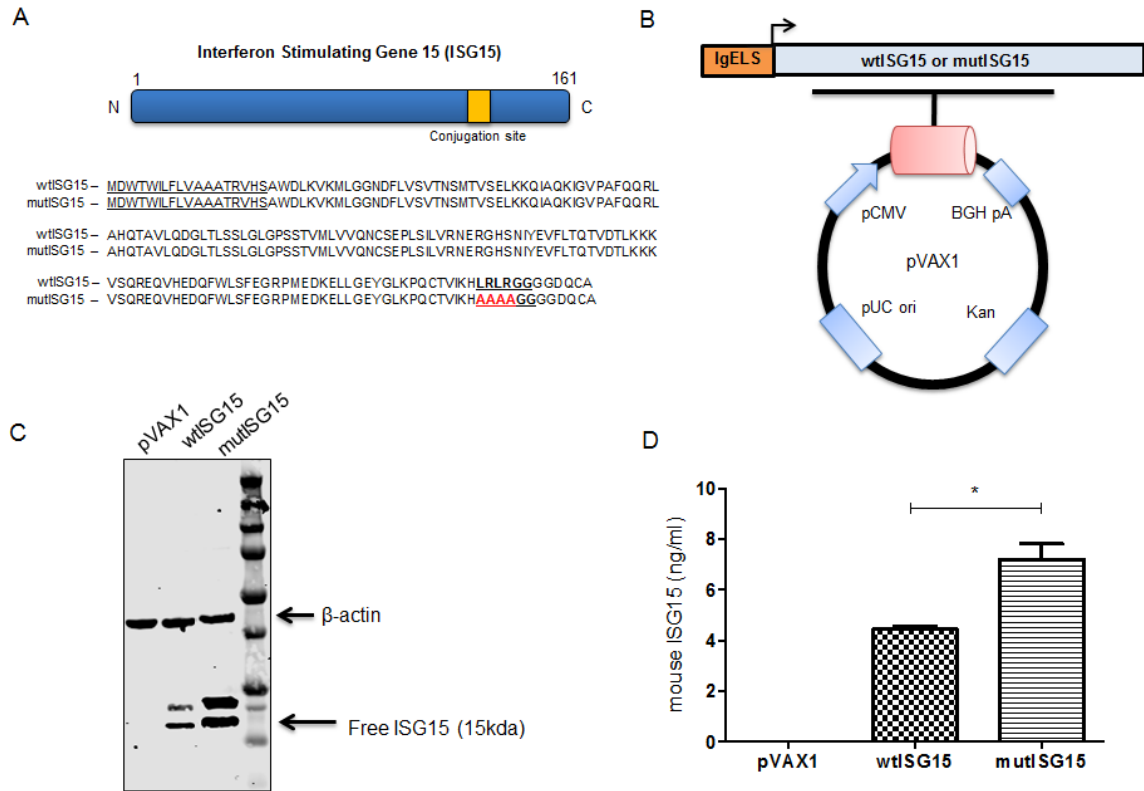


Figure 5.1. Generation and expression of ISG15 encoding DNA vaccine plasmids. (A) Schematic illustration of ISG15 protein and the amino acid sequences of wild-type ISG15 (wtISG15) and mutated ISG15 (mutISG15). The IgE leader sequences are underlined. The C-terminal ubiquitin-like conjugation site is bold and underlined. The mutation sites introduced into the conjugation motif for mutISG15 (unconjugated form) are in red. (B) map of ISG15 constructs. (C) Expression of ISG15 constructs examined by Western blot analysis. The lowest band represents free ISG15. (D) Detection of secreted wtISG15 and mutISG15 from transfected RD cells were confirmed via ELISA. Data represents the means with SEM for two replicate assays.

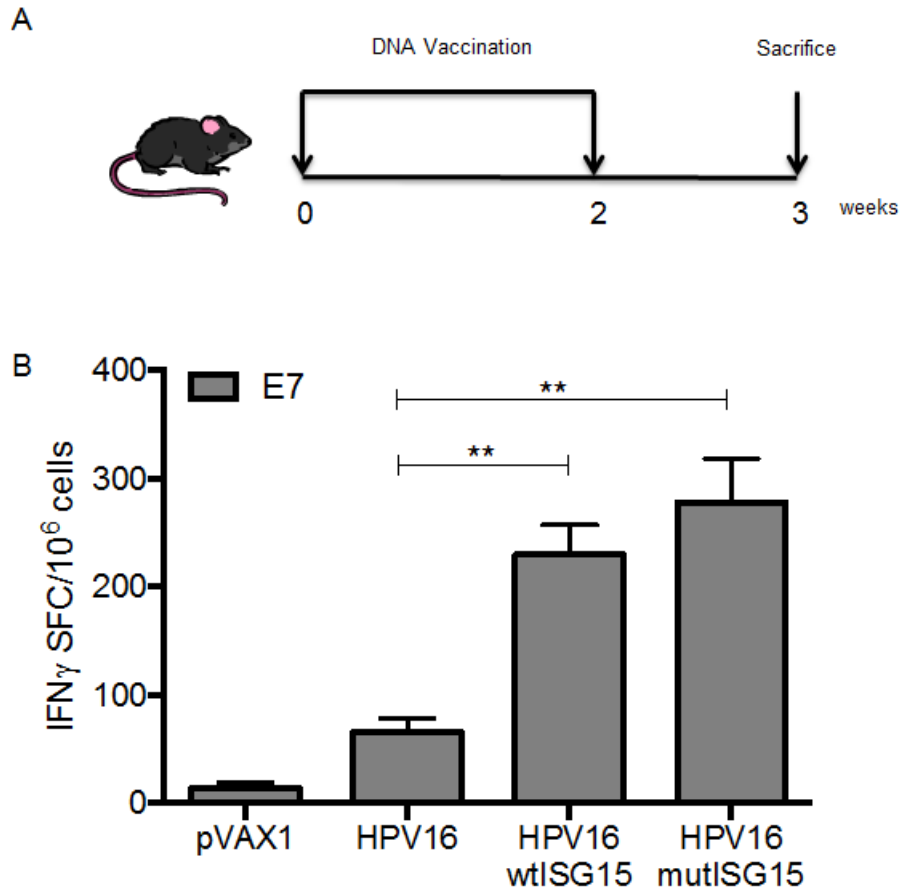


Figure 5.2. Co-delivery of ISG15 DNA vaccination promoted E7-specific CD8 T cell immune responses secreting IFN γ production. (A) Immunization schedule for DNA vaccine adjuvant study. C57BL/6 mice ($n = 4-5/\text{group}$) were immunized twice at two-week intervals with HPV16 construct with or without wtISG15 or mutISG15 adjuvant constructs via IM/EP delivery. One week after last vaccination, spleens were harvested to analyze the Ag-specific CD8 T cell responses. (B) The frequency of E7-specific IFN γ (spot forming cells/10⁶ splenocytes) responses induced after each vaccination was determined by IFN γ ELISpot assay in response to E7 pooled peptide containing the specific CD8 HPV16 E7 epitope (RAHYNIVTF). Data represent 2 independent experiments with 4-5 mice per group. *, $P < 0.05$; **, $P < 0.01$. Error bars indicate SEM.

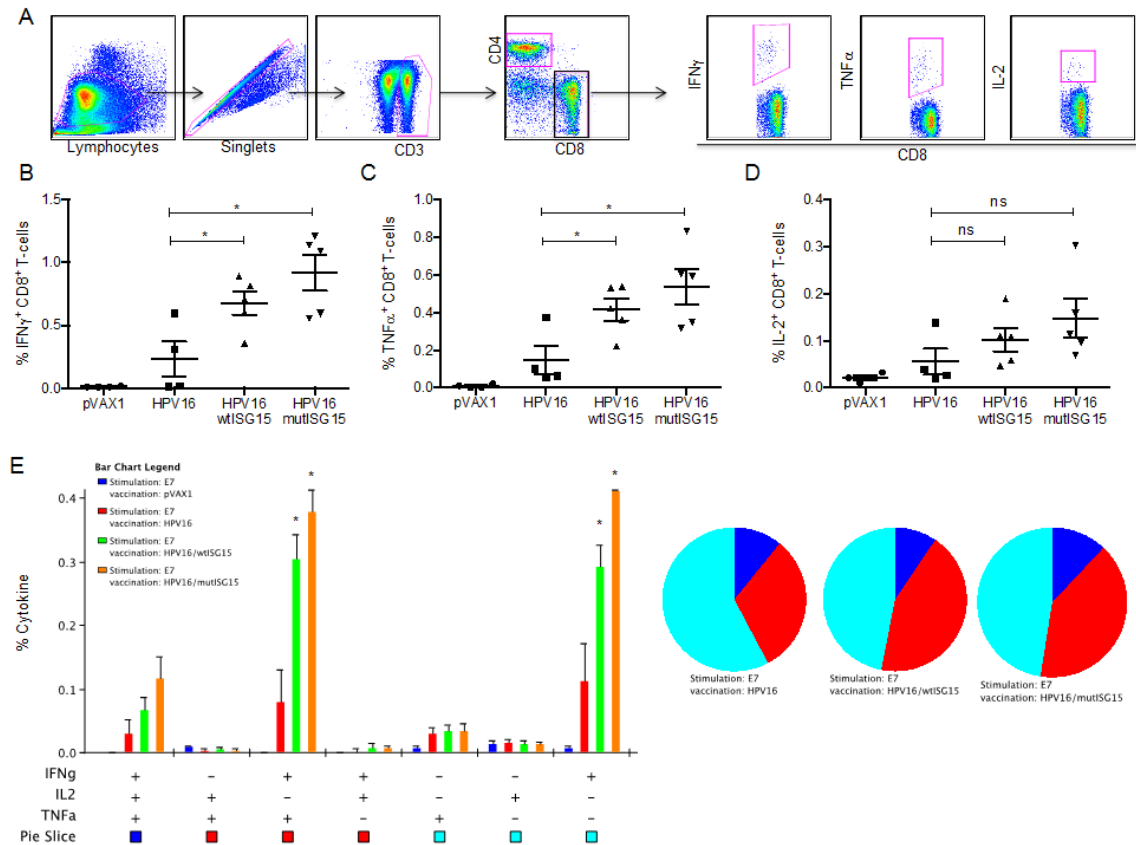


Figure 5.3. ISG15 induces polyfunctional HPV16 E7-specific CD8 T cells. (A) Schematic diagram of gating strategy used to identify Ag-specific CD8 T cell populations. (B-D). Column graphs show the percentages of HPV16 E7-specific CD8 T cells releasing total cytokines IFN γ (B), TNF α (C), and IL-2 (D) after stimulation with D^bE7₄₉₋₅₇-specific peptide. (E) Column chart show polyfunctional subpopulations of single-, double-, or triple-positive CD8 T cells releasing effector cytokines: IFN γ , TNF α , and IL-2 to E7₄₉₋₅₇-specific stimulation. Pie charts represent proportion of each cytokine population. Experiments were performed at least two times with similar results with 4-5 mice per group. *, P < 0.05 compared with HPV16 group. Error bars indicate SEM.

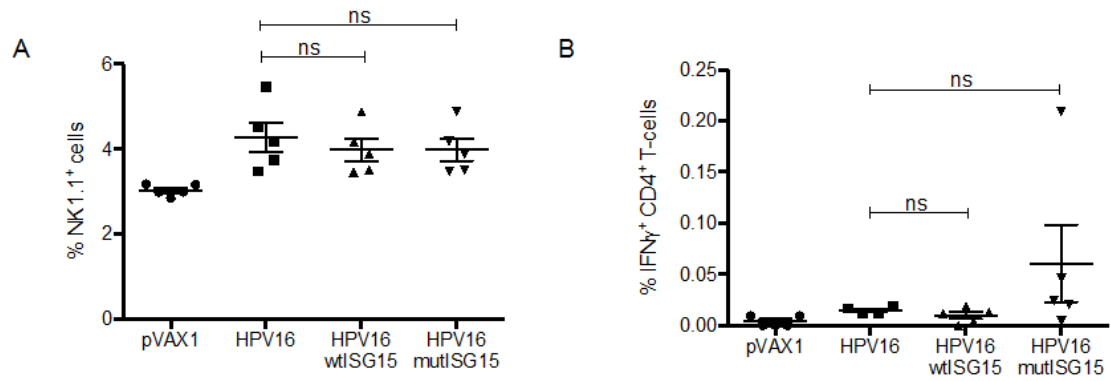


Figure 5.4. ISG15 had no profound influence on the NK or CD4 T cells. (A) Dot plot graphs show the percentages of NK cells in the spleens 1 week after final immunization with HPV16, HPV16/wtISG15 or HPV16/mutISG15 groups. (B) Dot graphs show HPV E7-specific CD4 T cells releasing IFN γ in response to *ex vivo* E7 pooled peptide stimulation in the spleens. Data was not significant. Experiments were performed at least two times (N = 4-5 mice/group). *, $P < 0.05$; **, $P < 0.01$. Error bars indicate SEM.

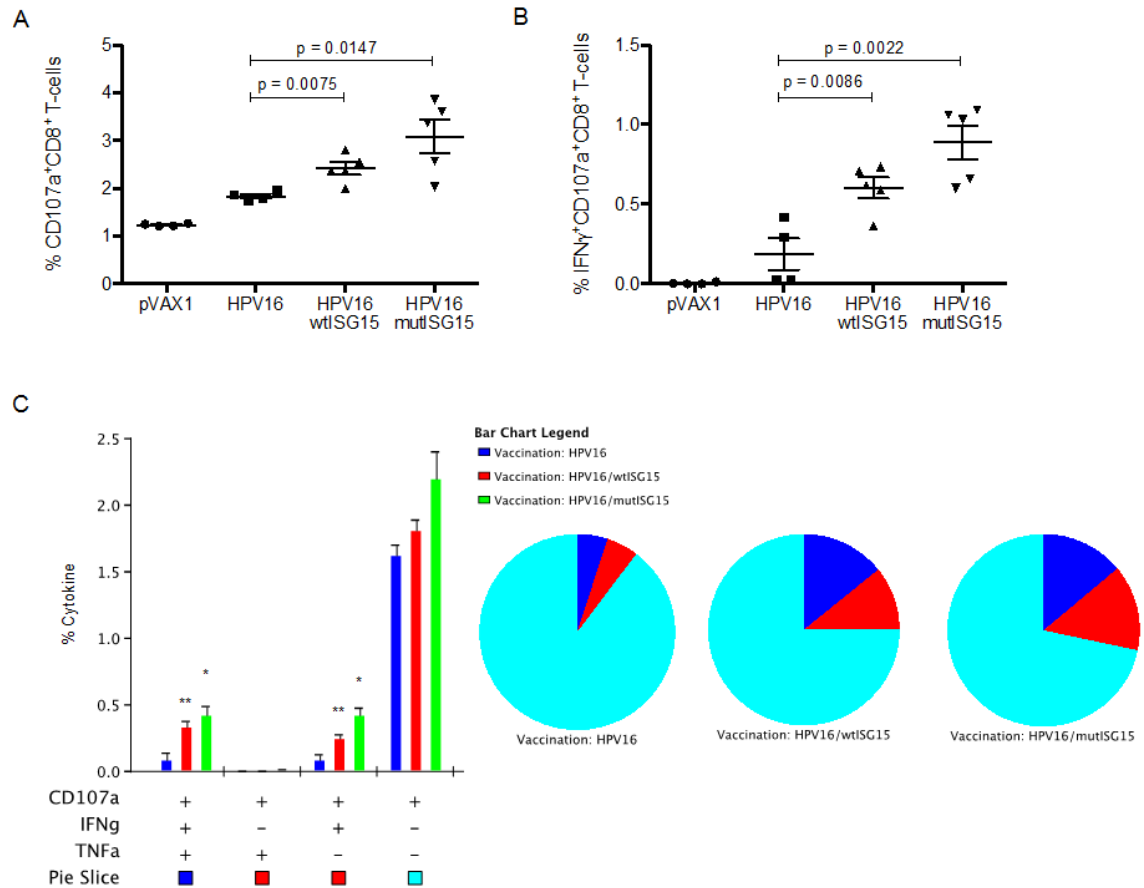


Figure 5.5. ISG15 induces HPV16 E7-specific CD8 T cells undergoing cytotoxic degranulation following immunization. E7-specific CD8 T cell responses measured by intracellular cytokine and CD107a staining after stimulation of splenocytes with D^bE7₄₉₋₅₇ restricted (CD8) peptide were examined in all groups of animals 1 wk after final immunization. (A) Ag-specific cytolytic degranulation of CD8 T cells measured by staining for degranulation marker expression, CD107a. (B and C) Column graph shows the frequency of cytolytic CD8 T cells simultaneously expressing only IFN γ (B) or the frequency of polyfunctional cytokine producing and/or CD107a expressing CD8 T cells (C). Experiments were performed at least twice with similar results with 4-5 mice per group. *, $P < 0.05$; **, $P < 0.01$ compared with HPV16 group. Error bars indicate SEM

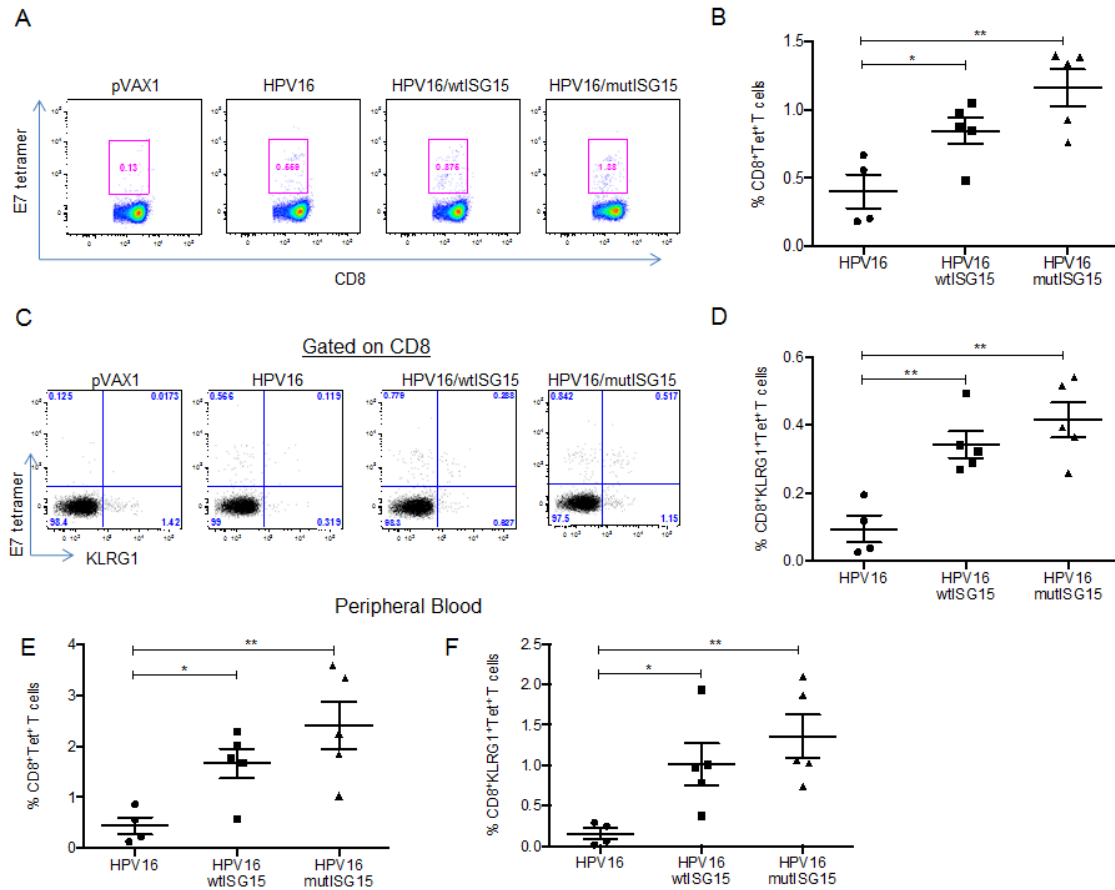


Figure 5.6. ISG15 augments the formation of the effector-memory E7-specific CD8 T cell population. Groups B6 mice (n = 4-5) were immunized twice with HPV16, HPV16/wtISG15 or HPV16/mutISG15 at two-week intervals. One week after last immunization, both splenocytes and peripheral blood mononuclear cells were strained for CD8, D^bE7₄₉₋₅₇ tetramer, and the effector memory KLRG1 marker. (A) Representative flow plot showing H2-D^b-RAHYNIVTF-restricted HPV16 E7-specific CD8 T cells in the spleen one week after final immunization, or (B) in data represented as a scatter plot graph. (C-D) Representative dot plots (C) and compiled data of the percentages of E7 tetramer-specific KLRG1⁺CD8⁺ effector memory phenotype population in the spleen (D). (E-F) The percentages of total D^bE7₄₉₋₅₇ tetramer-binding CD8 T cells from the peripheral blood (E) and tetramer-specific effector memory CD8 T cells (F). Data is representative of at least 2 experiments. *, $P < 0.05$; **, $P < 0.01$. Error bars indicate SEM.

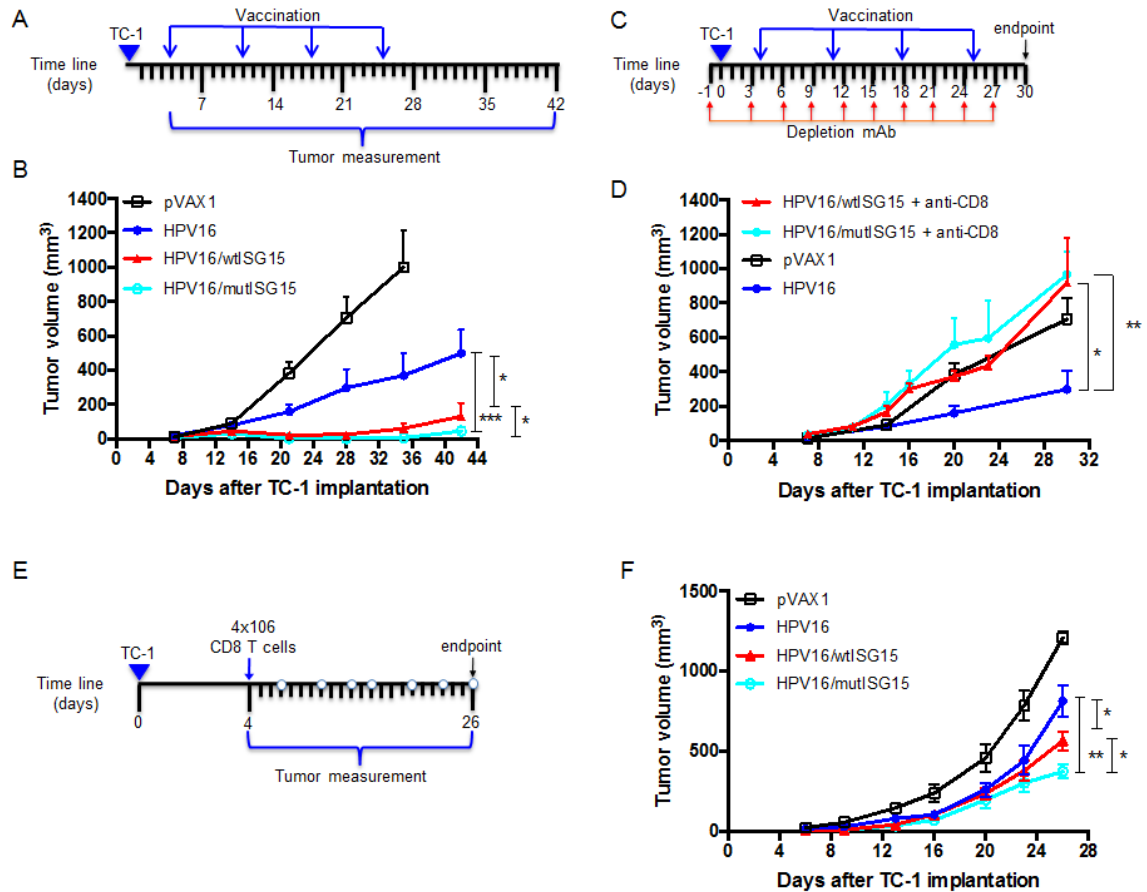


Figure 5.7. The therapeutic effects induced by ISG15 in tumor-bearing mice. (A) Schematic representation for therapeutic study. (B) Tumor growth measurement after therapeutic DNA/EP vaccination (n = 10). (C) Schematic representation for CD8 T cell depletion with therapeutic vaccination. (D) Tumor growth curve of vaccinated groups (n = 5) without CD8 T cells. (E and F) schematic representation for T cell adoptive transfer study (E). Approximately 4×10^6 CD8 T cells from vaccinated mice were purified from splenocytes and adoptively transferred into tumor-bearing T cell immunodeficient B6 Rag1 KO mice (n = 5) and assessed for tumor growth (F). All tumor-bearing mice were injected subcutaneously with 5×10^4 TC-1 cells. *, P < 0.05; **, P < 0.01; *, P < 0.001. Error bars indicate SEM.**

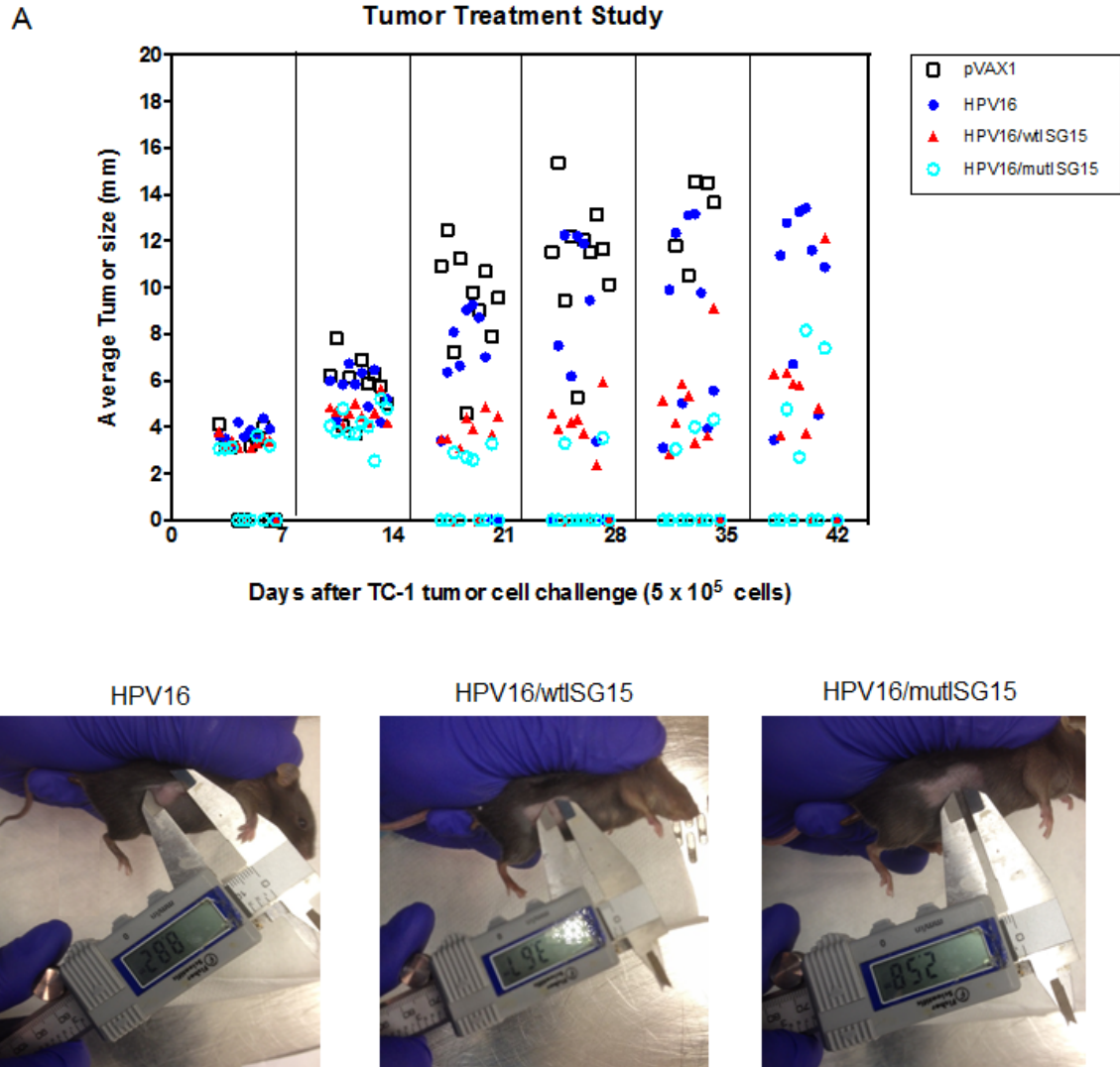


Figure 5.8. Inclusion of ISG15 as a vaccine adjuvant improves both tumor control and regression in tumor-bearing mice. Groups of C57Bl/6 mice ($n = 10/\text{group}$) were injected subcutaneously with 5×10^4 TC-1 cells. Starting on day 4 after tumor implantation, all groups of mice were immunized followed with three boosts at weekly intervals. Immunization with ISG15 constructs delayed tumor growth or led to tumor regression in tumor-bearing mice. Tumor measurements (average values for each individual mice) for each time point are shown only for surviving mice. Mice were sacrificed when tumor diameter reached approximately 18-20 mm. Images are representative examples of tumor size at day 42 after tumor implantation.

DISCUSSION

ISG15 is known to play a major role in antiviral defense. In addition, it has also been reported to function as an immunoregulatory molecule (6). The existence of a secreted unconjugated form of ISG15 has been reported to have cytokine-like activity, with evidence supporting its ability to induce IFN γ responses (9-13). Thus, this study builds on these findings and extends ISG15 research in a therapeutic tumor model system.

Herein, we first report the therapeutic efficacy of ISG15 immunoadjuvant properties to augment Ag-specific CD8 T cell tumor immunity. We used a well-established preclinical HPV therapeutic challenge model to test the adjuvant effects of ISG15 in a DNA vaccine setting. The main results of this study are that inclusion of ISG15 can (i) increase the polyfunctional Ag-specific CTL responses; (ii) induce effector-like memory CD8 T cell subset differentiation; (iii) have antitumor therapeutic effects; and (iv) elicit vaccine-induced protective immunity independent of conjugation, further establishing free ISG15 immunomodulatory properties.

In this study, we report that the inclusion of ISG15-induced robust Ag-specific IFN γ responses. We find this in accordance with previous studies indicating that ISG15 can induce IFN γ secretion by lymphocytes (7-10). However, we include new evidence that ISG15 delivered as a separate molecule in an adjuvant function, can drive CD8 T cells to enhance their secretion of IFN γ and TNF α , further adding to the pool of information regarding ISG15 immunomodulatory properties on T cells. It is noteworthy to report that in this model or approach, ISG15 was not involved in induction of NK cells, as previously shown (8). Furthermore, given that CTL functionality represents an important correlate of protective capacity against HPV16 established tumors (24,25,33), we report

that ISG15 promoted the expansion and Ag-specific cytolytic function of CD8 T cells by augmenting the expression of IFN γ , TNF α and the degranulation marker CD107a in various combinations. We also demonstrated that ISG15 delivered as a vaccine adjuvant, amplified E7 tetramer-specific CD8 vaccine-induced T cell responses. The reasons behind ISG15 ability to enhance the frequency of Ag-specific CD8 T cell responses are unknown. However, a study by Casanova and colleagues have shown that ISG15 may work in synergy with IL-12, suggesting that ISG15 likely promoted enhanced CD8 T cells induction and expansion synergistically with IL-12 (10). In addition, the ability of ISG15 to induce IFN γ secretion by lymphocytes, may also suggest that ISG15 might bind to a cell surface receptor to modulate immune responses (6). The identity of a cell surface receptor for ISG15 has yet to be discovered. Further studies will be needed to elucidate the mechanism(s) underlying the adjuvant effects of ISG15 including soluble ISG15.

The administration of immunoadjuvants in vaccines has long been studied as an important method of improving their efficacy, or potency. Here we explored the antitumor role of ISG15. Prior work in the TC-1 tumor challenge model, has demonstrated that this model is CD8 T cell dependent for protection (12,33-35). As expected, the results revealed that accordant with the enhanced polyfunctional CTL responses, administration of ISG15 led to strong inhibition of tumor growth, regression and prolonged survival. Moreover, depletion of CD8 T cells in mice nullified the antitumor effects of ISG15 and supported the tumors to grow larger compared to the non-treated CD8 mAb HPV16 group. These data support that the antitumor activity of ISG15 was dependent on CD8 T cells. Subsequently, as demonstrated in our adoptive transfer experiments, CD8 T cells alone can reduce tumor volume, suggesting that the ISG15

vaccine-induced CD8 T cell responses must be more functional at clearing HPV-infected cells. In this experiment setting, the enhanced induction of E7-specific effector memory-like T cells may have correlated with the therapeutic efficacy of ISG15-treated groups against established tumors. This notion is supported by the enhanced CTL activity (**Figure 5.4**) and E7 tetramer-positive T_{eff} cells in both the spleen and blood of mice vaccinated with ISG15 (**Figure 5.5**). Notably, there were more detectable CD8 T_{eff} cells responses in the periphery, suggesting trafficking of target specific CD8 T cells to the site of malignancy and initiating immediate effector function (26,31). Our studies appear to be supportive of previous work demonstrating that vaccines eliciting higher T_{eff} correlated with superior protective immunity against inhibiting tumor growth (1,5,14). Therefore, the magnitude and quality of E7-specific CD8 T cell memory population correlated with the efficacy of ISG15 treated groups to control or resolve tumors during the first 3 weeks of treatment. These potential correlates of immunity may represent a major tool for continued development of future tumor vaccines. Consistent with this is recent reports indicating that vaccine-induced effector memory may be the best prognostic factor for therapeutic vaccines targeting established tumors or latently infected pathogens (1,5,27,28). Central memory CD8 T cells elicited by ISG15 may have also been important, as central memory T cells are essential features by which vaccines can mediate protective immunity (29-31,36). This is an area of further investigation. Overall, on the basis of these findings, the improved therapeutic effect by ISG15 is associated with Ag-specific CTL responses.

In the same experimental setting, a highlight of this study was demonstrating that the protection afforded by ISG15 was most likely not dependent on its conjugated form, but rather on free ISG15. Our results indicated that mutISG15 was able to induce a similar trend of robust Ag-specific antitumor T cell responses compared to wtISG15,

suggesting that this activity was independent of ISGylation. The immunomodulatory property of soluble free ISG15 is in agreement with several studies suggesting that ISG15 acts as an immune activating cytokine (37). Both ISG15 constructs did not differ enormously in their effectiveness at eliciting E7-specific tumor immunity. Thus, given that both forms of the ISG15 constructs exerted similar immunostimulatory effects, the phenotypes in CD8 T cells were not dependent on the motif (38). Interestingly, mutISG15 was significantly better able to control the progression of established tumors compared to wtISG15-adjuvanted treated mice. As more mutant-ISG15 is secreted from transfected cells in our experiments (**Figure 5.1D**), it may orchestrate a more effective adaptive response; thus, leading to better control of tumor growth and pathogen clearance. However, the manner in which unconjugated secreted form of ISG15 (mutISG15) is able to induce better antitumor responses compared to wtISG15 is still not yet clear. Nevertheless, the ability of the mutISG15 form to induce superior antitumor responses highlights its potential to serve as an alternative potent ISG15 adjuvant. In addition, it emphasizes that developing new ways to increase the levels of free ISG15 may be a novel approach to treat cancer and other infectious diseases.

In summary, the results of the current preclinical study provide more insight into the immunomodulatory properties of free ISG15 and its potential to serve as a promising vaccine adjuvant in cancer immunotherapy. The results also confirm the notion that ISG15 can function as an immunomodulatory molecule. Moreover, the evidence that ISG15 can be an effective adjuvant to drive potent CD8 T cell responses, support future studies to evaluate its application in TB- or HIV-infection models.

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CHAPTER 6

ISG15 IS AN INFLAMMATORY MEDIATOR AND IMMUNOADJUVANT FOR INFECTIOUS DISEASE

ABSTRACT

The significance of the ubiquitin-like protein, interferon-stimulated gene 15 (ISG15), in human disease has gained considerably with studies that found ISG15 deficiency increases susceptibility to mycobacterial infection among other pathologies. However, the role of ISG15 in the cellular response to intracellular bacterial pathogens and the translation of its immunomodulatory properties to novel immunotherapies have not yet been explored. To accomplish this task, we employed the use of ISG15-deficient mice and *in vivo* expression vector to augment expression of murine ISG15 in wild-type mice. As with mycobacteria, ISG15^{-/-} mice are highly susceptible to infection with the model pathogen, *Listeria monocytogenes* (LM), and display significantly reduced production of IFN γ . Tissue-specific defects in adaptive immunity to LM were also observed in ISG15^{-/-} mice along with impaired dendritic cell maturation suggesting a possible role for ISG15 in T cell activation and memory formation. In fact, use of ISG15 as an immunoadjuvant enhances virus-specific CD8 T cells responses and provides protective immunity against lethal intracranial lymphocytic choriomeningitis virus (LCMV) challenge infection. This work elucidates the role of ISG15 as a critical mediator of innate anti-bacterial immunity and a potent activator of adaptive immunity, a finding with significant implications in immunotherapy development.

INTRODUCTION

Ubiquitin and ubiquitin-like proteins (UBLs) have essential roles in the development of functional immune responses (1). One ubiquitin-like protein, in particular, Interferon-stimulated gene 15 (ISG15), is gaining prominence due to its specific induction during infection and relevance in human disease (2,3). ISG15 is highly induced during viral infection and mediates protection against influenza, HIV, and Sindbis virus infection among others (4,5). Type I Interferon produced during an infection induces expression of ISG15, leading to its secretion and, like ubiquitin, conjugation to intracellular substrates through the action of a unique enzymatic cascade (6,7). While the molecular consequences of ISGylation are yet to be fully elucidated, it can inhibit protein ubiquitylation and, therefore, block viral particle release (8-15).

Recent studies, however, expand the scope of ISG15 function beyond innate antiviral defense (16,17). Bogunovic, et al. discovered that individuals with mutations in *Isg15* are more susceptible to infection by the intracellular bacterium *Mycobacterium tuberculosis* (Mtb). The increased susceptibility due to ISG15 deficiency in these patients correlated with impaired induction of pro-inflammatory cytokines by leukocytes (16,18). Interestingly, these data agree with *in vitro* studies previously performed over twenty years ago (19,20). In these studies, recombinant human ISG15 was found to activate leukocytes *in vitro* when added to culture media and induce production of proinflammatory cytokines (19). The use of a soluble form of ISG15 to induce these immune activating effects is relevant as several studies suggest that ISG15 may have immunomodulatory properties in its secreted form (18). Therefore, questions remain regarding the immunological functions of ISG15 beyond innate anti-viral immunity. This study endeavors to further elucidate this expanded role for ISG15 in immunity and translate its immunomodulatory properties to a novel therapeutic strategy.

METHODS

Mice

ISG15^{-/-} mice and their syngeneic wild-type strain C57BL/6J were obtained from Jackson Laboratory (Bar Harbor, ME) and bred and housed in the University of Pennsylvania Hill Pavilion Animal Facility and in the TTUHSC Abilene LARC. Mice were kept on a 12-hour light/dark cycle with sterile water and UV-treated or autoclaved standard rodent diet. All mouse experiments were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the TTUHSC and University of Pennsylvania according to the guidelines of the National Institute of Health.

Bacterial strains

LM strains were cultured in BHI (Brain-heart infusion, CM1135, Oxoid LTD, Hampshire, England) media supplemented with 50 ug/mL of streptomycin, harvested at mid-log growth phase (0.6-0.8 at O.D. 600), aliquots flash-frozen in liquid N₂, and stored at -80°C. LM stock titers were determined by serial dilution of a thawed stock vial, plating of dilutions onto BHI-streptomycin agar plates, and counting the colony-forming units (CFUs) after 18-24 hrs. at 37°C. For each infection experiment, a frozen stock vial was freshly thawed, bacteria pelleted by centrifugation, and the pellet washed twice with 1X phosphate-buffered saline (PBS).

***In vitro* LM Infection**

Infection of bone marrow-derived macrophage (BMM) was performed as described previously (61). For mRNA analysis, BMMs were seeded onto tissue culture-treated dishes, incubated overnight, and infected with LM at a multiplicity of infection (MOI) of 10. The infected cells were washed, gentamicin was added 30 min after infection, and

cells processed for RNA isolation using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions.

***In vivo* LM Infection**

For primary infection studies to determine cytokine responses by qPCR and ELISA, 6-8 week old C57BL/6 and ISG15^{-/-} mice were euthanized three days after intraperitoneal (i.p.) injection with 10⁵ CFU of LM in 200µl of sterile 1x PBS. To determine the role of ISG15 in poly(I:C)-exacerbated listeriosis, C57BL/6J and ISG15^{-/-} mice were infected i.p. with 10⁴ CFUs of LM alone or administered 150µg of poly(I:C) i.p. two days after LM infection. All mice were euthanized on day 4 post-infection and spleens extracted. Spleens were processed into single-cell suspensions, serially diluted, plated onto BHI-agar plates supplemented with 50ug/mL streptomycin and colony-forming units counted after overnight growth at 37°C. For longitudinal infection studies, 6-8 week old C57BL/6J and ISG15^{-/-} mice were i.p. injected with 10⁴ CFUs of LM in 200µl of sterile 1x PBS. At experiment end, mice were euthanized and processed for bacterial load. LM CFUs in the spleen and liver were determined as described previously (61). In **Figure 6.1**, mice were injected with 10³, 10⁴, and 10⁵ CFU LM in 200µl of 1x PBS i.p. and euthanized at day 4-post infection. Spleens and livers of the infected mice were harvested and LM bacterial load determined by serial dilution of single cell suspensions and colony-forming units counted after overnight growth on BHI-agar supplemented with 50ug/mL streptomycin. In **Figure 6.2**, 6-8 week old C57BL/6 and ISG15^{-/-} mice were infected with 10³ CFU of the attenuated $\Delta actA$ LM strain, DPL-4029 or virulent LM. These mice along with naïve WT and ISG15^{-/-} mice were subsequently challenged with intraperitoneal injection of 10⁵ CFUs LM. Five days after challenge, mice were

euthanized and organs processed for flow cytometric analyses and bacterial load determination.

Quantitative PCR

RNA was isolated from splenocytes and bone marrow-derived macrophages using an RNeasy plus mini kit (Qiagen). RNA was quantified using a Nanodrop spectrophotometer and 1ug of RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The Step One Plus Real Time system from Life Technologies was used for qPCR analysis in combination with FAST SYBR Green (Applied Biosystems). To determine relative quantity of target genes between groups, 18S rRNA was used as a reference.

ELISA

In **Figure 6.1**, serum was collected by post-euthanasia heart puncture bleeds and blood clots removed after incubation at 4°C for 30 minutes followed by centrifugation. Serum samples were diluted 1:40 and assayed for levels of IFN- γ using the mouse ELISA Ready-SET-Go! kit (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions. In **Figure 6.2**, splenocytes were stimulated with 5 ug/mL of H2-K^b-restricted LLO epitope peptide (62), MHC Class I-restricted HIV gag epitope peptide (63), or plate-bound anti-CD3/CD28 along with 50 U/mL of rhIL-2 overnight and media collected the following day. Undiluted conditioned media was assessed for IFN γ and TNF α using the mouse ELISA Ready-SET-Go! kit. Results were obtained at O.D. 450 using a Microplate reader (SynergyHT, BioTek) and analyzed on Gen5 (Ver1.08).

Flow cytometric analysis

In **Figure 6.1-6.2**, spleens were extracted from mice and placed in 5 mL complete media (Corning Cellgro; DMEM 1X; Cat no. 15-013-CM). Spleens were mechanically macerated and passed through 40 μ m cell strainers (Fisher, Cat no. 22363549, 22363547) to produce single-cell suspensions. Cells were treated with ACK lysis buffer for 3-5 minutes at room temperature and washed three times in 1x PBS. Cells were suspended in complete media and cell counts determined using a Beckman Coulter Vi-Cell XR. For T cell stimulation, 2×10^6 cells were plated in 96-well round-bottom plates and stimulated with 5 μ g/mL of MHC Class II-restricted LLO epitope peptide (NEKYAQAYPNVS) (64), MHC Class II-restricted E7 epitope peptide (DRAHYNI) (65), or PMA/ionomycin for 6 hours at 37°C, 5% CO₂ in the presence of monensin and 50 U/mL of rhIL-2. For cell surface staining, splenocytes were stained for various cell surface markers after Fc-blockade with anti-CD16/CD32(Clone 93; 14-0161-85) using fluorochrome-labeled mAbs (Supplementary information). All samples were acquired on an LSRII or LSRFortessa flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA), and data was analyzed using FlowJo software (v10, Tree Star). In **Figure 6.3**, splenocytes were added to a 96-well plate (1×10^6 /well) and were stimulated with the immunodominant LCMV CTL epitope (NP₃₉₆₋₄₀₄) (66) (Invitrogen) for 5-6 hours at 37°C/5% CO₂ in the presence of Protein Transport Inhibitor Cocktail (Brefeldin A and Monensin) (eBioscience) according to the manufacturer's instructions. The Cell Stimulation Cocktail (plus protein transport inhibitors) (phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin) (eBioscience) was used as a positive control and R10 media as negative control. All cells were then stained for surface and intracellular proteins as described by the manufacturer's instructions (BD, San Diego,

CA). Briefly, the cells were washed in FACS buffer (PBS containing 0.1% sodium azide and 1% FCS) before surface staining with flouochrome-conjugated antibodies. Cells were washed with FACS buffer, fixed and permeabilized using the BD Cytofix/Cytoperm™ (BD, San Diego, CA, USA) according to the manufacturer's protocol followed by intracellular staining. Antibodies and reagents for staining are listed in Supplementary information. All data was collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR) and SPICE v5.2 (free available from <http://exon.niaid.nih.gov/spice/>). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals. Dead cells were removed by gating on a LIVE/DEAD fixable violet dead cell stain kit (Invitrogen) versus forward scatter (FSC-A).

Plasmid construction

The GenBank accession no. Q64339.4 for mouse ISG15 was used to synthesize the ISG15 plasmid DNA construct. The ISG15 plasmid DNA construct has a highly efficient immunoglobulin E (IgE) leader sequence inserted at the 5'end of the gene. The construct was commercially synthesized and genetically optimized (codon- and RNA-optimization) for expression in mice and then subcloned (all by GenScript, Piscataway, NJ) into a modified pVAX1 mammalian expression vector (Invitrogen, Carlsbad, CA). Plasmid expressing pLCMV-NP (NP) was prepared as previously described (34).

Transfection and expression of plasmids

In vitro ISG15 was confirmed by western blot (WB) analysis. 293T cells were cultured in a 6-well plate and transfected with construct using Neofectin transfection reagent (NeoBiolab) following manufacture's protocol. Forty-eight hours later, cells were lysed

using modified cell lysis buffer (Cell Signaling) with complete protease inhibitor cocktail tablets (Roche) and cell lysate was collected. WB analysis was performed with an anti-ISG15 antibody (Cell Signaling) and visualized with IRDye 800CW goat anti-rabbit antibody (Li-Cor) using the Odyssey imaging system (Li-Cor). β -actin served as a loading control and visualized with IRDye 680 goat anti-mouse antibody (Li-Cor). In addition, an indirect immunofluorescence microscopy assay was also executed to confirm expression of ISG15 DNA construct. Rhabdomyosarcoma (RD) cells were plated on two-well chamber slides (BD Biosciences) and grown to 70% confluence overnight in a 37°C incubator with 5% CO₂. The cells were transfected with 1 μ g of ISG15 constructs and the control plasmid pVAX (1 μ g/well) using TurboFectinTM8.0 Transfection Reagent (OriGene) according to the manufacturer's instructions. Forty-eight hours later the cells were fixed on slides using ice cold methanol for 10 min. The cells were stained with anti-ISG15 mouse monoclonal antibody (Cell Signaling) and subsequently incubated with Alexa 555-conjugated anti-rat secondary antibody (Cell Signaling). Slides were mounted using Fluoromount G with DAPI (Southern Biotechnology). Images were analyzed by fluorescence microscopy (Leica DM4000B, Leica Microsystems Inc, USA) and quantification was conducted using SPOT Advanced software program (SPOTTM Diagnostic Instruments, Inc). Secretion of ISG15 was examined by using a CircuLex mouse ISG15 ELISA kit (MBL International), according to manufacturer's protocol.

Vaccinations and LCMV challenge

Mice were immunized once intramuscularly (i.m.) in the tibialis anterior muscle as previously described (29,67). *In vivo* electroporation was delivered, with the CELLECTRA adaptive constant current electroporation device (Inovio Pharmaceuticals), at the same site immediately following vaccination as described (67). The mice ($n = 5$)

were immunized with either 10 µg pVAX1 or 10 µg pLCMV-NP with or without 11 µg of ISG15 construct. All studies were repeated at least three times. For lethal challenge studies, mice were challenged i.c. with 40xLD₅₀ of LCMV Armstrong as previously described [34] in 30 µl of virus diluent (PBS with 20% FBS and 1X Anti-Anti (Invitrogen, Carlsbad,CA). All mice LCMV challenged were housed in a BSL-2 facility and were observed daily for 21 days.

ELISpot assays

Spleens were harvested 21 days following immunization to monitor vaccine-induced responses as previously described (29,67). After spleens were harvested and processed, IFN γ ELISpot assays were performed to determine the antigen-specific cytokine secretion from immunized mice as described previously (29,67). The LCMV-specific T cell responses were assessed by stimulating splenocytes with the immunodominant H2-D^b LCMV CTL epitope (NP₃₉₆₋₄₀₄) (66).

Statistical analysis

The one-tailed student *t* test was applied for comparison of the quantitative data of the cellular immune responses induced by infection or vaccination. Statistically significant outliers were removed from datasets by the ROUT method. All error bars indicate SEM and all tests were performed using the Prism Software (La Jolla, CA) (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001). Survival curves were analyzed by log-rank (Mantel-Cox) test.

RESULTS

To determine the relevance of ISG15 in the innate immune response to the model pathogen, *Listeria monocytogenes* (LM), ISG15 gene expression was inspected after LM infection in wild-type C57/BL6 mice. *Isg15* mRNA expression was significantly induced at the peak of infection on day 3, with 100-fold higher expression of *Isg15* in the spleen, a major site of infection, compared to uninfected control mice (**Figure 6.1A**). Infection of bone marrow-derived macrophages (BMM) with LM also resulted in a time-dependent induction of *Isg15* and the gene encoding the ISG15 E1 conjugating enzyme, Ube1L (**Figure 6.1B**). Furthermore, secreted ISG15 protein could be detected in the serum of infected WT mice at the peak of LM infection but not in ISG15^{-/-} controls (**Figure 6.1C**). Expression of *Isg15* during LM infection was Type I IFN-dependent as antibody-mediated blockade of IFN- β significantly blunted the *Isg15* induction in LM-infected BMMs (**Figure 6.1D**). These data suggest the ISG15 pathway is induced during LM infection and is dependent on production of Type I IFN. In contrast to viral infection, Type I IFN exacerbates certain bacterial infections including listeriosis by impairing both innate and adaptive responses to LM (21-23).

To determine if ISG15 mediates Type I IFN exacerbation of listeriosis, WT and ISG15^{-/-} mice were infected with LM and Type I IFN was induced by administering a dsRNA mimetic molecule, poly (I:C) (21,24). Surprisingly, ISG15 is not necessary for Type I IFN-mediated exacerbation of listeriosis and two independent experiments suggested that ISG15^{-/-} mice may even be more susceptible to LM infection (**Figure 6.1E**). The role of ISG15 in innate immunity to LM was further explored with a time-course infection. On day 1 post-infection, ISG15^{-/-} mice were more resistant to acute infection with LM as evidenced by significantly reduced bacterial burden (**Figure 6.1F**). However, bacterial burden was significantly elevated in ISG15^{-/-} mice at the peak of

infection on day 3 and continued to rise subsequently in contrast to their wild-type counterparts. We next sought to determine if this result was only relevant at the initial infection dose, 10^4 CFUs, as previous studies have shown dose-dependent susceptibility to LM (25). WT and ISG15^{-/-} mice were infected with a log range of infection doses from 10^3 CFUs to 10^5 CFUs of LM. In the spleens of WT mice receiving the lowest dose of LM (10^3 CFUs), only 40% of mice had evidence of listeriosis (**Figure 6.1G**). However, 80% of ISG15^{-/-} mice had detectable levels of LM in their spleen (**Figure 6.1G**). Similar results were observed in the livers of WT and ISG15^{-/-} mice after receiving the lowest dose with 20% and 100% of mice demonstrating listeriosis, respectively (**Figure 6.1H**). Significantly increased listeriosis was also observed at higher starting doses in both the spleens and livers of ISG15^{-/-} mice (**Figures 6.1G-H**). While NK cell numbers were similar (**Figure 6.1I**), the increased susceptibility to acute LM infection in ISG15^{-/-} mice did correlate with significantly reduced expression of splenic *ifng* and serum levels of IFN γ (**Figure 6.1J-K**), an essential proinflammatory cytokine in the clearance of LM infection (26,27).

As LM virulence has previously been reported to inversely correlate with adaptive immunity to LM (23), we sought to determine if ISG15 deficiency also compromised adaptive immune responses to LM. After prior vaccination with an attenuated strain of LM (DPL-4029), with reduced ability to undergo ActA mediated cell-to-cell spread, WT and ISG15^{-/-} mice were challenged with the virulent 10403S strain of LM and development of adaptive immunity assessed on day 5 post-infection (**Figure 6.2A**). Both WT and ISG15^{-/-} demonstrated similar induction of LM-specific CD8 T cell immunity in the spleen as evidenced by specific production of IFN γ and TNF α after H2-K^b LLO-epitope₂₉₆₋₃₀₄ peptide stimulation (**Figure 6.2B**). In addition, ISG15^{-/-} mice generated

similar numbers of LLO-specific splenic CD4 T cells after LM challenge and stimulation with LLO₁₉₀₋₂₀₁ epitope peptide (**Figure 6.2C**). Furthermore, no deficiencies were observed in overall splenic CD4 and CD8 T cell populations of ISG15^{-/-} mice during primary and secondary responses to LM (**Figure 6.2D-E**). As expected, splenic LM bacterial load in previously infected mice was comparable between ISG15^{-/-} and WT mice (**Figure 6.2F**). However, liver bacterial load in previously infected ISG15^{-/-} mice was approximately 100-fold greater than in previously infected WT mice (**Figure 6.2G**). Similar liver-specific defects in adaptive immunity were observed when WT and ISG15^{-/-} mice were first infected with a low dose of virulent LM (10³ CFUs) prior to high-dose challenge (**Figure 6.2H-J**). While these results suggest ISG15 may be involved in liver immune-privilege (28), the altered adaptive immune response to LM may be due to defects in the myeloid compartment. In fact, greater numbers of myeloid cells were observed in the spleens of ISG15^{-/-} mice during LM infection, but there were significantly fewer conventional dendritic cells to facilitate induction of a robust T cell response to control infection in the liver (**Figure 6.2K-M**). Furthermore, stimulation of splenic dendritic cells with LPS revealed that ISG15 deficiency impairs dendritic cell maturation as evidenced by reduced surface levels of costimulatory molecules CD80 and CD86 (**Figure 6.2N-P**). Collectively, these results would suggest that ISGylation facilitates T cell priming, a function that has not yet been attributed to it. Therefore, ISG15 may be important in fostering cell-mediated adaptive immunity by augmenting antigen-presenting cell number and function.

The observed results, that ISG15 deficiency impairs IFN γ responses during LM infection, led us to determine if these observed immunoregulatory properties of ISG15 could be leveraged to enhance responses to immune-privileged sites. Therefore, we

induced overproduction of ISG15 in wild-type mice by delivering it as a DNA vaccine adjuvant to augment T cell-mediated immunity. This *in vivo* mammalian ISG15 expression DNA plasmid was developed as previously described (29) (**Figure 6.3A**). Briefly, the mouse ISG15 gene was cloned into the pVAX mammalian expression vector under the control of a CMV promoter and with an IgE leader sequence to allow for secretion (**Figure 6.3A**). After transfection of 293T cells with pVAX-mISG15, cells proficiently expressed intracellular murine ISG15 as determined by Western Blot analysis and fluorescent microscopy (**Figures 6.3B** and **6.3C**, respectively). As shown in **Figure 6.3C**, ISG15 expression was found in the cytoplasm as determined by colocalization with the nuclear stain, DAPI (30,31) (**Figure 6.3C**). Due to the inclusion of an IgE leader sequence, transfection with pVAX-mISG15 also resulted in proficient secretion of soluble mouse ISG15 into the culture supernatants from pVAX-mISG15 transfected 3T3 cells in comparison to empty vector transfected cells (**Figure 6.3D**). To determine if ISG15 could augment CD8 T cell-mediated immunity *in vivo*, further studies were performed with an infection model in which ISG15 is not relevant, the intracranial (i.c.) lymphocytic choriomeningitis virus (LCMV) infection model (32,33). The LCMV infection model is an established model to study CD8 T cell responses to the brain of infected mice (34). Therefore, to characterize the CD8 T cell responses driven by ISG15, groups of mice were intramuscularly administered plasmid expressing LCMV structural protein, NP, with or without plasmid expressing mISG15. Mice receiving the NP vaccine administered with mISG15 generated significantly more D^bNP₃₉₆₋₄₀ (NP396)-specific IFN- γ spot-forming cells (SFCs) than mice receiving pVAX-NP or empty pVAX plasmid alone (**Figure 6.3E**). Furthermore, the NP vaccine administered with mISG15 induced significantly higher percentages of NP-specific polyfunctional CD8 T cells compared to mice receiving vector expressing antigen alone (**Figure 6.3F**). To determine if the

observed adjuvant effect of mISG15 impacted survival, mice were challenged 21 days after vaccination with a lethal intracranial dose ($40 \times \text{LD}_{50}$) of the LCMV Armstrong strain (34). Dramatically increased survival to lethal LCMV infection was observed in the mISG15 and NP vaccine group compared to the group-receiving NP antigen alone (**Figure 6.3G**). Taken together, these data demonstrate that mISG15 can act as an immunoadjuvant to activate highly effective antigen-specific CD8^+ T-cell responses to even immune-privileged sites.

FIGURES

FIGURE 6.1

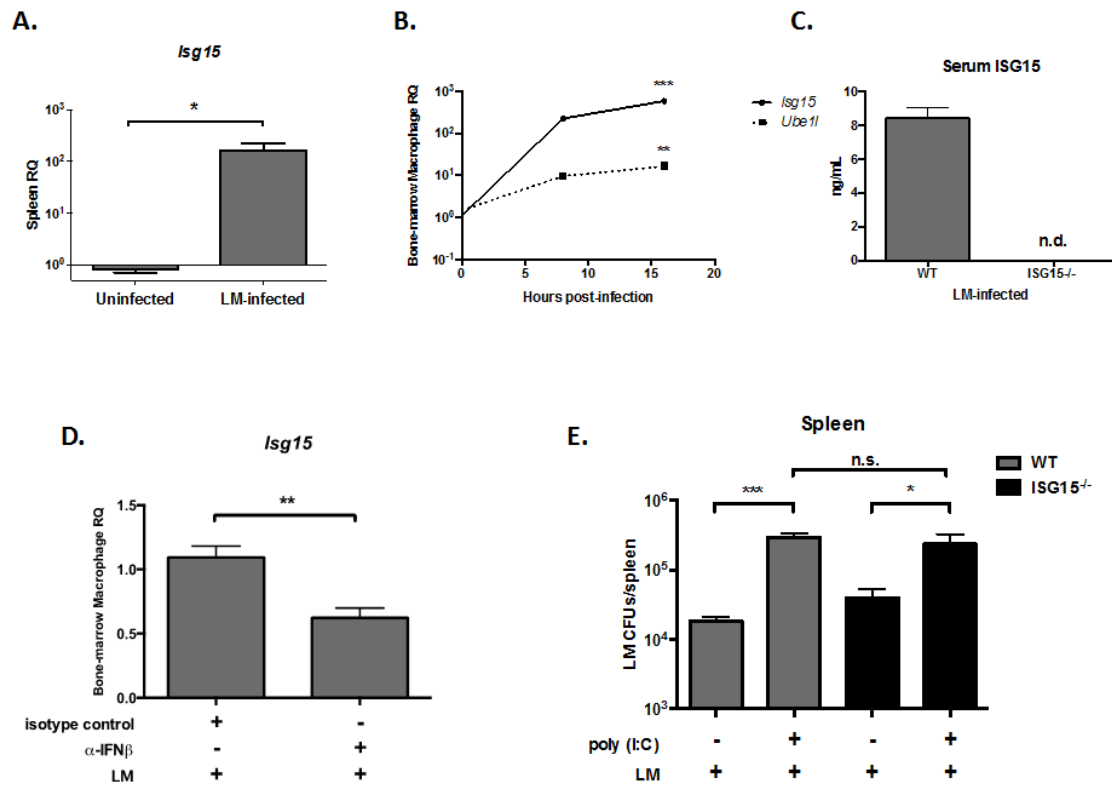


FIGURE 6.1 CONT.

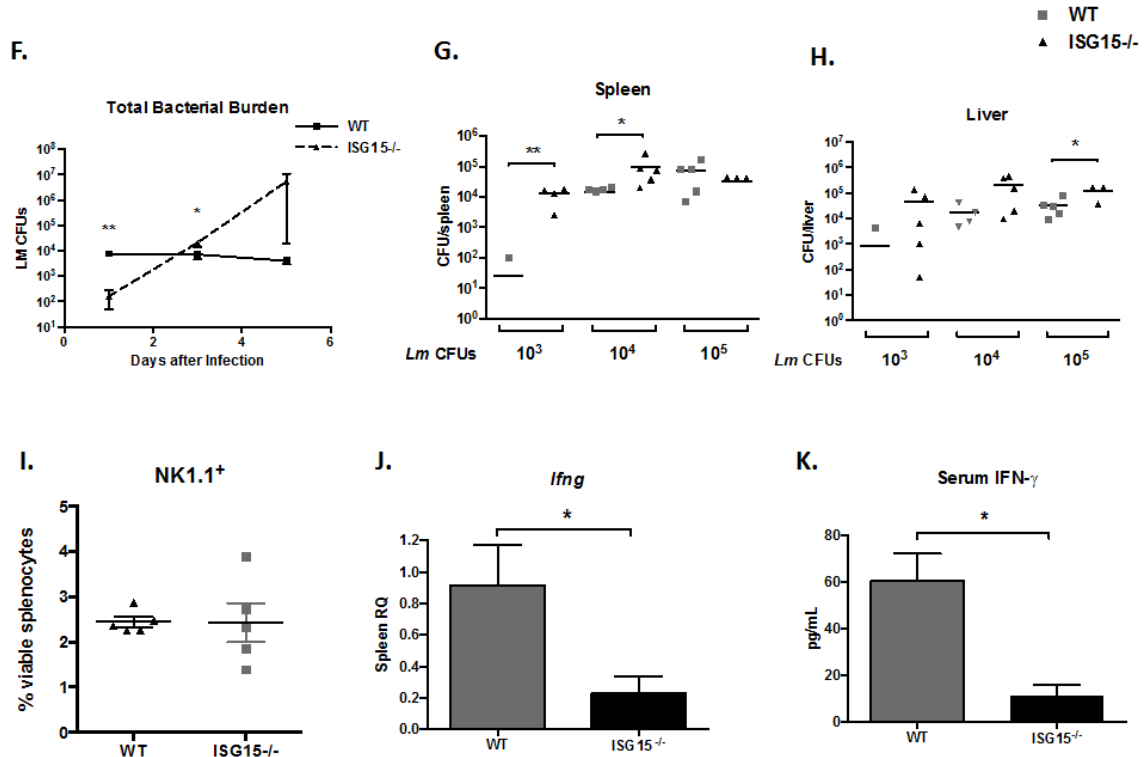


Figure 6.1. ISG15 deficiency impairs innate immune responses to LM (A) Mice (n=3/group) were infected with 10^5 LM CFUs and euthanized at the peak of infection on day 3 along with a group of uninfected mice. Spleens were excised, processed into a single-cell suspension, and RNA extracted. After conversion to cDNA, spleens were assessed for expression of *Isg15* by qPCR analysis. Bar graphs depict induction of *Isg15* expression after LM infection. (B) Bone marrow-derived macrophage (BMM) were differentiated with M-CSF and infected with LM (n=3/group). BMM were lysed after 8 and 16 hours post-infection along with uninfected controls and processed for RNA extraction. After cDNA conversion, BMMs were assessed for *Isg15* gene expression along with the gene for its E1-activating enzyme, *Ube1l*. (C) WT (n=4) and ISG15^{-/-} mice (n=5) infected with 10^5 LM CFUs were euthanized on day 3 post-infection and serum collected to assess levels of secreted ISG15 protein by ISG15 ELISA. (D) BMMs were infected with LM followed by treatment with isotype control or IFN- β blocking antibody one hour post-infection (n=3/group). At experiment end, BMMs were lysed, mRNA extracted, converted to cDNA, and *Isg15* gene expression assessed by qPCR analysis. (E) WT (n=4) and ISG15^{-/-} (n=5) mice were infected i.p. with 10^4 CFUs of LM alone or administered 150 μ g of poly(I:C) i.p. two days after LM infection (n=3/group). All mice were euthanized on day 4 post-infection and spleens excised and processed into single-cell suspensions. Suspensions were serially diluted and plated out on BHI-streptomycin agar plates in order to determine colony-forming units (CFUs) per spleen. (F) WT and ISG15^{-/-} mice (n=3/group) were infected i.p. with 10^4 CFUs of LM and euthanized on day 1, 3, and 5 post-infection. Spleens and livers from infected mice were excised and processed into single-cell suspensions. Suspensions were serially diluted and plated out on BHI-streptomycin agar plates in order to determine colony-forming units (CFUs) per organ. Total bacterial load was determined by adding LM CFUs from the spleen and liver of each mouse. In order to determine if susceptibility to LM infection is dose-dependent, WT and ISG15^{-/-} mice (n=5/group) were infected i.p. with a log range of doses of LM CFUs. At day 4 post-infection, spleens and livers were extracted, processed into single-cell suspensions, serially diluted, and plated out on BHI-streptomycin agar plates. (G) Scatter plots depicting LM CFUs in the spleens of WT and ISG15^{-/-} after infection with a log range of doses. (H) Scatter plots depicting LM CFUs in the livers of WT and ISG15^{-/-} after infection with a log range of doses. (I) Scatter plot depicting NK1.1⁺ splenocytes in WT and USP18^{-/-} mice during primary LM infection with 10^5 CFUs. (J) WT (n=5/group) and ISG15^{-/-} mice (n=3/group) were infected i.p. with 10^5 CFUs of LM and euthanized at the peak of infection on day 3 post-infection. Spleens were excised, processed into single-cell suspensions, and RNA extracted. After conversion to cDNA, spleens were assessed for expression of the proinflammatory cytokine gene *Ifng*. (K) Production and secretion of IFN- γ was confirmed by ELISA analysis of serum from WT and ISG15^{-/-} mice (n=5/group) infected with 10^5 CFUs LM at peak of infection on day 3 post-infection. Amount of IFN- γ protein in serum was calculated with a protein standard. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 6.2

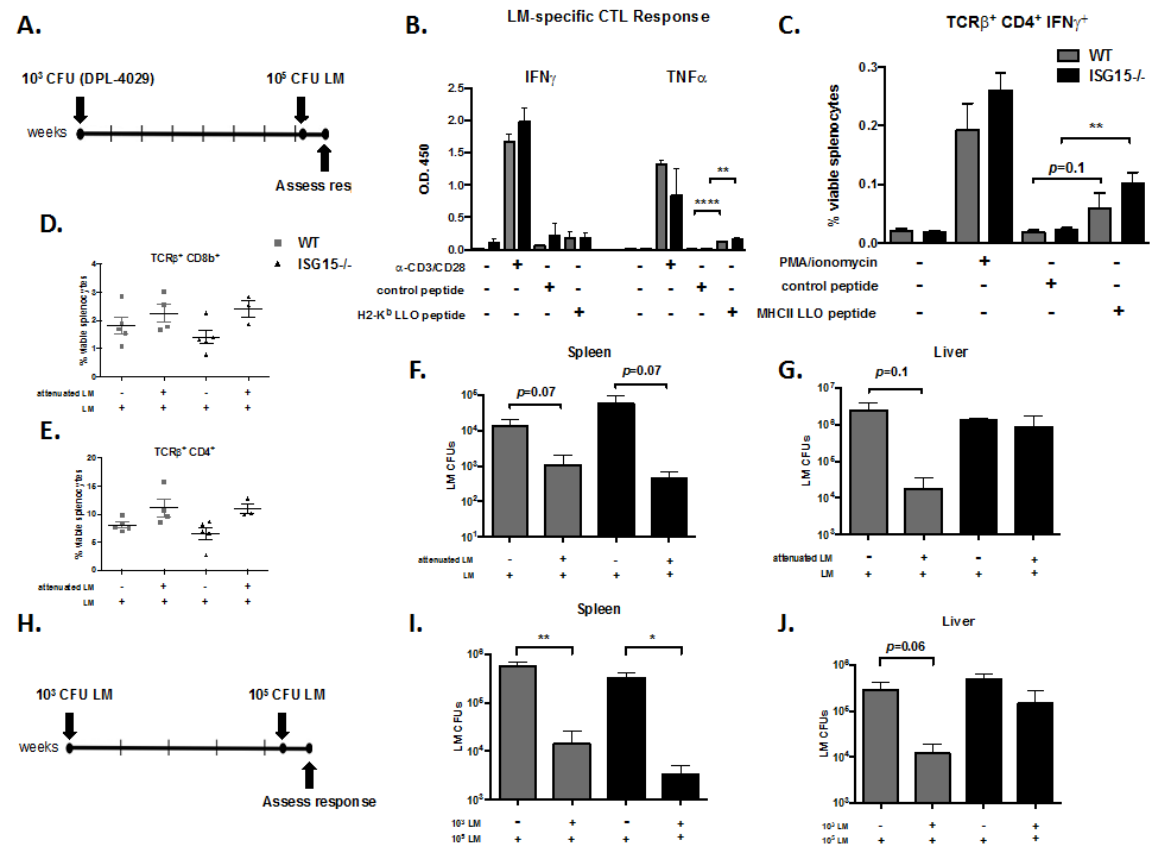


FIGURE 6.2 CONT.

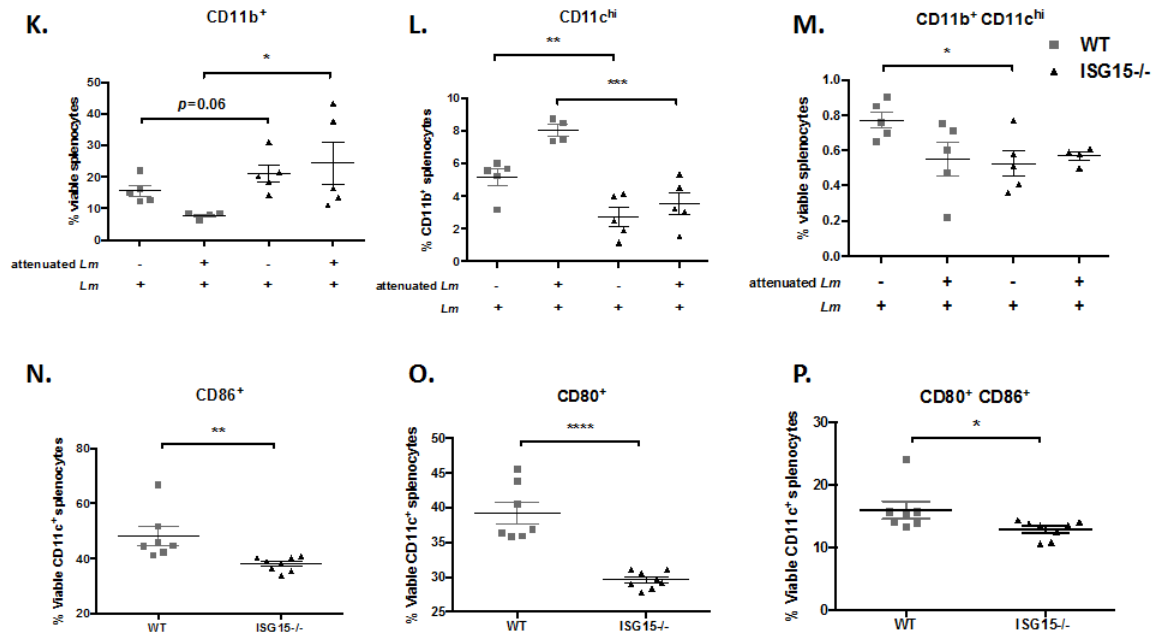


Figure 6.2. ISG15 deficiency alters adaptive immune response to LM and impairs DC maturation. (A), Schematic depicting the experimental procedure to determine the role of ISG15 in adaptive immunity to LM. Briefly, WT and ISG15^{-/-} mice were infected i.p. with 10^5 CFUs of LM with or without prior infection with 10^3 CFUs of the attenuated LM strain, DPL-4029 (B), Production of IFN γ and TNF α by LLO-specific CD8⁺ T cells after stimulation with MHC Class I-restricted LLO epitope peptide, control peptide, or anti-CD3/CD28 as measured by ELISA after prior infection with DPL-4029 and challenge with LM. (C), LLO-specific IFN γ -producing splenic CD4⁺ T cells from LM-infected WT and ISG15^{-/-} mice with stimulation by phorbol 12-myristate 13-acetate (PMA) (50ng/mL) and ionomycin (800ng/mL), control peptide or MHC Class II-restricted LLO epitope peptide. (D), Splenic CD8⁺ T cell and (E), splenic CD4⁺ T cell percentages during innate and adaptive response to LM. (F), LM bacterial burden in the spleen and (G), liver of WT and ISG15^{-/-} mice. (H), Schematic depicting alternate experimental procedure to determine the role of ISG15 in adaptive immunity to LM. Briefly, WT and ISG15^{-/-} mice were infected i.p. with 10^5 CFUs of LM with or without prior infection with 10^3 CFUs of the virulent LM strain, 10403S. (I) LM bacterial burden in the spleen and (J), liver of WT and ISG15^{-/-} mice from experiment depicted in H. (K), Splenic myeloid cells during innate and adaptive response to LM from experiment depicted in Fig. 2A. (L), Percentage of myeloid cells that are CD11c^{hi} and (M), overall percentage of splenocytes that are conventional DCs during LM infection. (N), WT and ISG15^{-/-} spleens were stimulated with 1 μ g of LPS for 6 hrs. and surface expression of markers associated with DC maturation were assessed by flow cytometry for expression of CD86, (O), CD80 and (P), co-expression of CD80 and CD86. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$.

FIGURE 6.3

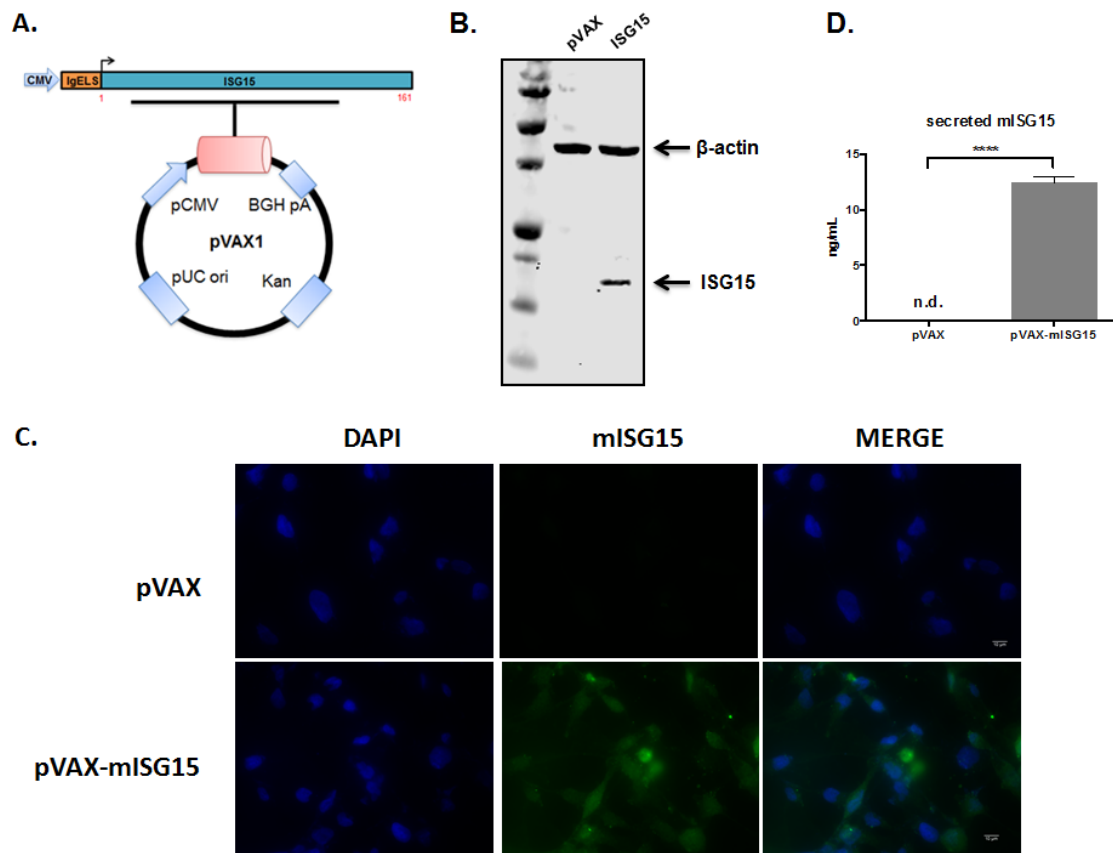


FIGURE 6.3 CONT.

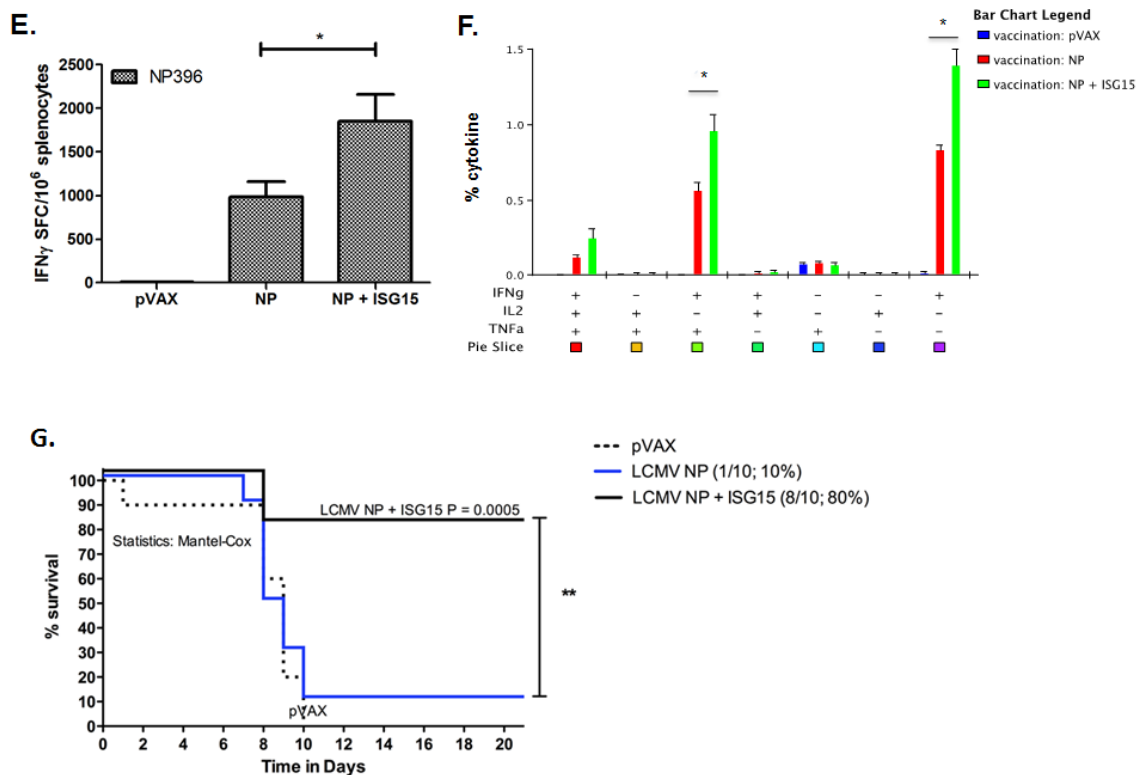


Figure 6.3. Elevated ISG15 expression augments virus-specific CD8 T cell responses and increases survival against lethal LCMV challenge. (A), Depiction of ISG15 protein adjuvant constructs. (B), expression of ISG15 in 293 T cells as examined by Western blot analysis. Labeled panels show protein detected by anti-ISG15 mAb. (C), Detection of expression of ISG15 via immunofluorescence microscopy. (D), secretion of ISG15 after transfection of empty pVAX and pVAX-mISG15 in 3T3 cells (n=3/group) was confirmed via ELISA of conditioned media. (E), B6 mice (n=5/group) were immunized once with or without ISG15 and 21 days later mice were sacrificed and spleens were processed to monitor the vaccine induced immune responses. IFN γ ELISpot was performed to detect antigen specific immune responses to the LCMV DbNP396-404 antigen (NP396) in combination with ISG15 when used in IM immunization via electroporation. (F) Multiparameter flow cytometry was used to determine the percentages of polyfunctional CD8⁺ T cell cytokine profile. The bar chart shows the percentage of NP-specific CD8⁺ T cells displayed as triple, double or single positive CD8⁺ T cells secreting cytokines. (G) Mice (n=10/group) were immunized one time IM using EP with 10ug of empty vector control plasmid (pVAX) or 10ug of LCMV-NP with or without ISG15 adjuvant. At day 21 post-vaccination, mice were challenged intracranial with 40xLD50 LCMV and animal survival is displayed in the graph. Experiments were performed at least three times in independent experiments and data are representative of the results. *, $P < 0.05$; **, $P < 0.01$, ****, $P < 0.0001$.

DISCUSSION

ISG15 has a well-established role in innate antiviral responses, but recent studies have shed light on further functions in immunity for this small ubiquitin-like protein (3,16,17). Our studies support this expanded role of ISG15 in immunity, as we find it is essential for innate immunity to the bacterial pathogen, LM (**Figure 6.1**). Furthermore, while ISG15 deficiency does not hinder formation of splenic LM-specific T cell responses, protective adaptive immunity is not evident in the liver of ISG15^{-/-} mice after subsequent LM challenge (**Figure 6.2**). Additionally, splenic DCs from ISG15^{-/-} mice have reduced expression of maturation markers and overexpression of ISG15 by WT mice was able to augment pathogen-specific CD8 T cell responses and increase survival to lethal intracranial LCMV challenge (**Figure 6.3**). These results confirm a growing consensus that the role of ISG15 is likely more pleiotropic than just directing anti-viral innate immunity and prompt further questions regarding its mechanisms in immunity and promise in immunotherapy development (18).

In regard to innate defense against LM infection, previous studies suggest ISG15 may impact several stages of the bacterium's lifecycle. Once LM invades a phagocytic cell, autophagy activates and facilitates clearance and processing of the pathogen (35,36). ISG15 deficiency has previously been reported to impair autophagy and, therefore, may exacerbate listeriosis and hinder presentation of LM-derived epitopes (37). However, LM has evolved strategies to evade autophagic destruction and escape into the host cell cytosol (38-40). Once LM invades the cytosol of an infected cell, it rapidly polymerizes actin and propels itself into the cell membrane, forming protrusions into surrounding cells and propagating the infection (41-44). ISG15 may also hinder this process as it covalently binds actin and regulates its polymerization, possibly hindering LM virulence at this stage (45,46). Lastly, protrusion formation by LM, a necessary step

in its cell-to-cell spread, is carried out through exploitation of the ezrin/radixin/moesin (ERM) complex, an interface between the cytoskeleton and the cell membrane (47,48). ISG15 conjugates components of the ERM complex and, while the consequence of this ISGylation is unclear, it may hinder its usefulness for LM cell-to-cell spread (30).

More broadly, our findings in ISG15^{-/-} mice provide support for recent studies demonstrating that individuals with mutations in *Isg15* are at increased risk for intracellular facultative bacterial infection (16,49). This susceptibility is likely due, in part, to reduced IFN γ production in ISG15^{-/-} mice and leukocytes from ISG15-deficient patients leading to impaired macrophage activation (16). In fact, reduced activation of ISG15^{-/-} macrophage has previously been reported in the context of viral infection (50). One possible explanation for the role of ISG15 in IFN γ production could be that it contains alarmin-like activity (51). Over two decades ago, a pair of studies found that recombinant ISG15 alone, when added to culture with PBMCs, could stimulate IFN γ production (19,20). Further credence to this alarmin hypothesis is that ISG15 is expressed and secreted to a high degree during disease states such as infection and cancer (10,52-56). Alternatively, recent studies suggest that ISG15 may augment the ability of a cell to produce IFN γ in response to stimulus (16,17). This may be the consequence of a recently proposed mechanism for ISG15, stabilization of the negative regulator of Type I IFN signaling, USP18 (17). In response to intracellular bacterial infection, USP18 facilitates robust STAT4-dependent IFN γ production and bacterial clearance (17,57). Interestingly, mutation of the LRGG repeat in ISG15 did not impair ISG15-mediated stabilization of USP18 suggesting conjugation is not necessary for some ISG15 functions (17). Further studies to inspect the dependence of ISG15 immunomodulation on USP18 status and conjugation potential are ongoing.

In addition to an expanded role in innate anti-bacterial immunity, our study also provides evidence for a novel role for ISG15 as a powerful immunoadjuvant for T-cell mediated immunity (**Figure 6.2-6.3**). While ISG15 deficiency appeared to have a limited impact on T-cell responses to LM, overexpression of ISG15 significantly augmented polyfunctional LCMV-specific CD8 T cell responses, which are essential to survival against LCMV challenge (58). An explanation for this discrepancy could be that endogenous ISG15 is not essential for intrinsic T cell function, as there could be redundancy within the Type I IFN-induced responses. Thus, there could be other mechanisms by which the T cells are still able to get activated even if it is to a lesser extent. This needs to be further investigated. On the contrary, in the case of the LCMV model, ISG15 clearly had an effect on augmenting CD8 T cells when delivered as an immunoadjuvant. This suggested that CD8 T cells, on the other hand, could respond to soluble ISG15. We find this in accordance with previous data demonstrating that free soluble ISG15 can induce lymphocytes to induce IFN γ responses (16,17). However, evidence is still lacking as to whether ISG15 binds a receptor to mediate these immunomodulatory CD8 T cell effects or if intracellular uptake and conjugation is required. Further work is required to elucidate the mechanism of action between secreted vs. non-secreted ISG15. We are currently investigating this approach. Alternatively, our results provide strong evidence that ISG15 may facilitate or orchestrate the adaptive responses through enhanced dendritic cell maturation (**Figure 6.2**). One possible hypothesis is that ISG15 is acting as a DAMP much like HMGB1 that binds a pattern-recognition receptor (PRR) to induce inflammation (59,60). A previous study by Padovan, et al. in 2002 suggested a similar DC stimulatory property for ISG15 secreted from melanoma cells (54). More recently, ISG15 was found to synergize with IL-12 to

enhance activation of Mtb-specific T cell responses (16). The summation of this evidence suggests that ISG15 is a powerful immunoadjuvant for CTL-mediated immunotherapy. Therefore, further studies are warranted to determine the possibly broad applicability of ISG15 as an immunoadjuvant for other diseases and to explore the relevance of ISG15 conjugation and secretion in this novel role.

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CHAPTER 7

DISCUSSION, IMPLICATIONS, AND FUTURE STUDIES

“The reward for work well done is the opportunity to do more.”

– Jonas Salk

Vaccines represent one of the greatest medical interventions against diseases. However, there is still a great need for effective vaccines against cancers and many chronic infectious such as HIV, tuberculosis and malaria. For these pathogens, it is known that CD4 T helper 1 (T_H1) and CD8 T cell-mediated immunity is critical in preventing or controlling the onset of disease (1). These challenging diseases have shifted vaccine focus in generating T cell-mediated immune responses and adjuvants that specifically drive this preferred effects (1). Adjuvants are critical components of most clinical vaccines which are used to shape the quantity and quality of immune responses. Currently available U.S. Food and Drug Administration (FDA)–licensed adjuvants are poor inducers of T_H1 and even worse at treating CD8 T-cell responses (2,3). Therefore, it is important to identify a new generation of potent vaccine adjuvant(s) that can induce potent and durable T cell immunity.

Different vaccine platforms have been studied, but the development of DNA-based vaccines in conjunction with molecular adjuvants has emerged as particularly promising for enhancing effective T cell induced viral and tumor immunity (4). It is becoming increasingly well recognized that the administration of immunostimulatory molecules, such as cytokines or cytokine-like molecules, to modulate immune responses has great potential in medicine (4,5). Thus, by harnessing the power of our own immune system, cytokines are promising vaccine adjuvants for enhancing the immune responses against an array of infectious diseases. Several studies into the biology of IL-33 and ISG15 suggest that they might serve as effective vaccine adjuvants (6,7). In this thesis, we show for the first time the efficacy of both IL-33 and ISG15 as DNA vaccine adjuvants in various models of infection. We show new insight into the biological properties of both IL-33 and ISG15, and highlight their potential as new promising adjuvants at improving the magnitude and function of effector CD8 T cell responses.

Therefore, both IL-33 and ISG15 are new players in the immunoadjuvant arena, with the potential to improve effectiveness of future immunotherapies for cancer and chronic diseases. Collectively, our results provide strong evidence for their future role as potent novel adjuvants in vaccines designed to boost CD8 T cell immunity

IL-33 as a Vaccine Adjuvant

IL-33, a member of the IL-1 cytokine family is released by necrotic cells or by activated innate immune cells during tissue damage or infection (6,8). It is considered to serve as the first line of defense against pathogens, in other words, to serve as an alarmin, by providing an endogenous danger signal that triggers inflammation and promotes cell-mediated immune response. For many years IL-33 has been studied in the context of T helper type 2 (T_H2)-driven inflammatory disorders (9). Interestingly, IL-33 has now emerged as a cytokine with a plethora of pleiotropic properties. Depending on the immune cells targeted by IL-33, it is reported to not only promote T_H2 immunity, but evidence has begun to unveil IL-33's unappreciated role to induce both T_H1 and CD8 T cell-mediated immunity (6,10). Therefore, in this study we investigated the potential role of IL-33 to act as an immunoadjuvant to elicit effective CD8 T cell-mediated tumor and viral immunity.

Herein, we first demonstrate that both biologically active forms of IL-33, full-length (proIL-33) and mature IL-33 (mtrIL-33) when combined with a HPV16 E6/E7 encoded DNA vaccine, enhanced the Ag-specific effector memory CD8 T cell immune responses. Their adjuvant activities skewed toward the T_H1 axis, and not to the T_H2 axis. We reported that both isoforms drove $IFN\gamma$ responses, but neither form drove high secretion of IL-4 or any elevation of IgE levels as previously reported (11,12). Finally,

both proIL-33 and mtrIL-33 adjuvants induced potent anti-tumor CD8 T cell immunity which facilitated successful tumor regression of established TC-1 tumor-bearing mice. Given these significant findings that IL-33 can augment bonafide Ag-specific CD8 protective tumor immunity, we expanded the scope of these studies to assess its efficacy in a LCMV viral challenge model. The LCMV model is known to require CD8 T cell responses for protective immunity (13-18). For these studies I utilized mtrIL-33, instead of proIL-33, given proIL-33 alternative action as a nuclear transcription factor which still needs to be further elucidated (6,8,9). Thus, I only focused on IL-33's cytokine activity and noted that mtrIL-33 delivered as an adjuvant significantly enhanced the LCMV-specific antiviral immunity and provided complete or significant protection against a high-dose lethal LCMV intracranial challenge. Collectively, these studies validated IL-33's role as a potent vaccine adjuvant for future T cell vaccine studies.

The specific role in the protective responses against HPV-associated cancers and LCMV infection have been attributed to CD8⁺ T cell immune responses, and therefore, are the focus for achieving effective immunity against such diseases (13-21). Our results showed that the cytokine secreting T cell responses induced by proIL-33 and mtrIL-33 were mainly mediated by polyfunctional Ag-specific CD8 T populations secreting IFN γ ⁺TNF α ⁺IL-2⁺, IFN γ ⁺TNF α ⁺ and IFN γ ⁺, while a large proportion of CD8 T cells having a cytolytic phenotype. Although we found this data in accordance with Bonilla *et al.* demonstrating that IL-33 can drive plurifunctional CD8 T cell responses in a viral infection model (10), we further demonstrated that the delivery of IL-33 isoforms as immunoadjuvants can indeed enhance plurifunctional CD8 T cell responses, further expanding the pool of information we now know about IL-33. In addition, we reported that both IL-33 isoforms could significantly amplify and increase the magnitude of

tetramer-specific effector CD8 T cell responses in the periphery in a vaccine setting, which correlated with superior viral protection and tumor regression (Chapters 2 & 3). The reasons behind the role of IL-33 to expand the frequency of CD8 T cells are not yet entirely clear (10,22,23). Recently, Luzina *et al.* demonstrated in a vaccine delivery approach that mtrIL-33's effect is ST2-dependent (11). Moreover, substantial research has shown (i) that activated CD8 T cells can express ST2 receptor and (ii) that ST2 is important for IL-33 action, as mice deficient in ST2 are entirely unresponsive to IL-33 (24). Therefore, the ability of ST2⁺CD8 T cells to respond to immunoadjuvant proIL-33 and mtrIL-33 is a possible explanation for the vaccine-induced augmentation (10,22). On the other hand, although one might assume that proIL-33 should induce similar effects to mtrIL-33 because they bind to the same ST2 receptor, Luzina *et al.* also reported that they have differences in their specific activities. Luzina *et al.* demonstrated that proIL-33 promotes inflammation differently from mtrIL-33 in an ST2-independent fashion (11). Additional studies that involve IL-33 receptor knockout mice will help confirm whether our proIL-33 T_H1 adjuvant properties are dependent or independent of ST2 receptor. We are currently pursuing these studies. These data demonstrate the overall superiority of immunoadjuvant IL-33 in enhancing the Ag-specific CD8⁺ T cells in a DNA vaccine setting.

Another mechanism behind IL-33's potential to drive the formation and differentiation of effector memory CD8⁺ T cells is likely due to its ability to significantly increase T-bet expression in CD8⁺ T cells *in vivo* (Chapters 2 & 3). Joshi and colleagues have shown that overexpression of T-bet is enough to induce the formation of KLRG1⁺ effector cells and that CD8⁺ T cells lacking T-bet are impaired in forming these cytolytic effector cells (25). Therefore, my data presented in this thesis supports the notion that an increase in T-bet is associated with effector CD8⁺ T cell differentiation and their ability

to have CTL phenotype. This finding also supports previous data showing by Yang *et al.* demonstrating that CD8 T cells treated with IL-33 in the presence of IL-12 result in T-bet-dependent expression of ST2 *in vivo* (22). Furthermore, it is also possible that our selected vaccine antigens, which are potent T_H1 antigens, may have facilitated the induction of T_H1 cytokines IL-12 and IFN γ secretion by APCs. Therefore, this induced favored T_H1 cytokine milieu perpetuated by our selected antigen may have induced a favorable immune environment that allowed IL-33 to foster a greater T_H1 cell-mediated immune response. Further studies are needed to understand exactly how IL-33 induces T_H1 IFN γ responses in the context of an *in vivo* immune setting.

Overall, my studies conclude that IL-33 can act as an immunoadjuvant to increase the potency of DNA vaccines. We established that some of the preclinical beneficial effects of IL-33 as an adjuvant are: (i) IL-33 can increase the seroconversion rate; (ii) facilitate the use of smaller doses of antigen to induce protective immunity; (iii) reduce the number of doses required to achieve protection; (iv) provide an appropriate desired immune response (T_H1 and CD8 T cells); (v) increase the generation of effector memory T cells; and (vi) increase the speed of initial response. In the future, we hope to determine if IL-33 can also improve weak immune responses, where it will be beneficial for the elderly or immunocompromised individuals. Taken together, the improved efficacy of our DNA vaccines offered by IL-33 in this thesis highlights its potential utility as a future vaccine adjuvant in the treatment of a variety of chronic diseases, including in the context of cancer vaccine therapies.

IL-33: T_H2 vs T_H1 Route of Immunization

Although IL-33 originally has been associated with T_H2 immunity, our IL-33 studies report that IL-33 delivered as a vaccine adjuvant could modulate immune responses toward a T_H1/CD8⁺ T cell response. Under different conditions it appears that IL-33 can have different functions either associated in driving T_H2- or T_H1-immune responses when delivered *in vivo* (10,11,26,27). How one method elicits T_H2 responses and another T_H1 response is unclear. The reasons for the differences may be attributed to variations in the routes of immunization in which the surrounding microenvironment (targeting cells and cytokine network) potentiate different outcomes. For instance, delivery into the mucosal sites which is normally rich with T_H2-driven cells (T regulatory cells, mast cells, basophils, eosinophils and innate lymphoid cells) may favor a T_H2 response, while it is likely that residential and local immune cells in the muscle may favor a T_H1 response during DNA vaccination. However, the key cell types in the muscle site that could be expressing ST2 and responsible for mediating the vaccine-induced responses have not been investigated. Furthermore, the T_H1-driven nature of the adjuvant properties could have also been perpetuated because of the selected vaccine target antigen activating APCs to trigger IL-12, which will support IL-33's IFN γ -inducing activity (22), and without it, a T_H1 response will dominate. Therefore, further testing is needed to determine if IL-33 is dependent on IL-12 to favor a T_H1 response *in vivo*. Overall, this supports the theory that the immunomodulatory functions of IL-33 might actually be more complex than initially assumed. Therefore, the route of delivery should be carefully studied to maximize the desired T cell phenotype in a particular immune setting to better target a particular disease

IL-33 Isoforms as Future Vaccine Adjuvants

Along with all the different established cytokines, there has recently been an appreciation for alternative splicing or processing of cytokine genes (eg. IL-1, IL-2, IL-7, IL-18), which result in multiple cytokine isoforms with different functional activities (28). Cytokine isoforms can provide additional diversity to their complex biological effects that participate in control and protection against different foreign pathogens. Indeed, IL-33 has emerged as a cytokine containing several different isoforms that can play a role in facilitating protective immunity (11,27-31). Tsuda *et al.* demonstrated the existence of multiple splice variants of IL-33 dependent on the cell type expressing IL-33 (30). Furthermore, Lefrancais *et al.* reported that neutrophil-specific proteases in neutrophils could cleave full-length human IL-33 to generate several mature processed variants with enhanced biological activity (tenfold) compared with proIL-33 (28). They also demonstrated that murine IL-33 can be similarly cleaved by these same neutrophil proteases (cathepsin G and elastase), generating two isoforms of mtrIL-33 (28). In our studies, in chapter 3, two bands of IL-33 were detected after transfection with proIL-33 in human rhabdomyosarcoma cells (27). Therefore, the observed humoral responses elicited by our proIL-33 adjuvant, may not have been driven solely by proIL-33, but also by either spliced variants of proIL-33 or processed forms of proIL-33 (aka mtrIL-33), as processing of the adjuvant did occur *in vitro*. These alternatively spliced or processed IL-33 cytokine isoforms represent attractive candidates for further study as possible vaccine adjuvants or immune modulating therapeutics. For future studies we plan to compare the immune modulation induced by these different identified isoforms of proIL-33 (splice variants) and mtrIL-33 (processed forms). This will provide insight into their potential to be properly utilized for different vaccination systems. Thus, fully understanding the IL-33 signaling system, especially its cytokine contributions as a

nuclear modulator versus extracellular molecule, will open new chapters on how to harness the power of all IL-33 isoforms in future vaccines and cancer therapies. The accessibility and continued study of both ST2 and IL-33 knockout mice will help enhance our understanding of IL-33's ability to influence adoptive immunity and its power to protect against disease.

ISG15 as a Vaccine Adjuvant

Given our success with IL-33 as vaccine molecular adjuvant to induce potent T cell immunity, we decided to continue this successful approach to identify additional adjuvants capable of amplifying CD8 T cell antitumor and antiviral immunity. I therefore, employed a DNA vaccination approach to investigate the inclusion of ISG15 to also enhance Ag-specific CD8 T cell responses.

ISG15 is an ubiquitin-like protein induced by type I interferon and is associated with antiviral activity (32). For decades, free ISG15 has been implicated in the production of IFN γ , thus functioning as an immunomodulatory molecule (33-35). More recently, a study by Casanova and colleagues confirmed this cytokine-like role for ISG15 by demonstrating that ISG15-deficiency was associated with a loss of IFN γ , which in turn led to increased susceptibility to mycobacterial disease in both mice and humans (36). However, ISG15's role to influence CD8 T cell responses and act as a vaccine adjuvant has not yet been explored. In this study, we showed for the first time the efficacy of ISG15 as a molecular adjuvant to augment Ag-specific CD8 T cell viral and tumor immunity. We used the well-established preclinical LCMV intracranial and HPV-associated therapeutic challenge models to test the adjuvant effects of ISG15 in a DNA vaccine setting. The main results of this study are that inclusion of ISG15 can (i)

increase the polyfunctional Ag-specific CTL responses secreting $\text{IFN}\gamma^+\text{TNF}\alpha^+\text{IL-2}^+$; (ii) induce effector-like memory CD8 T cell differentiation; (iii) have antitumor therapeutic effects; (iv) induce significant LCMV protective immunity; (v) elicit vaccine-induced protective immunity independent of conjugation, further establishing free ISG15 perhaps functions as a cytokine; (vi) increase the magnitude and phenotype of tetramer-specific CD8 T cell responses, which correlated with tumor regression; and finally that (vii) CD8 T cell depletion and adoptive transfer experiments revealed that ISG15 protective efficacy was CD8 T cell-mediated. Collectively, my work provides more insight into the immunomodulatory properties of ISG15 and its potential to serve as an effective CD8 T cell vaccine adjuvant in a therapeutic tumor or infectious setting.

The reasons behind ISG15 ability to enhance the frequency of Ag-specific CD8 T cell responses are unknown. However, a recent study by Casanova and colleagues have shown that ISG15 may work in synergy with IL-12, suggesting that ISG15 likely promoted enhanced CD8 T cells synergistically with IL-12 (36). In addition, the ability of ISG15 to induce $\text{IFN}\gamma$ secretion by lymphocytes, may also suggest that ISG15 might bind to a cell surface receptor to modulate immune responses (32). The identity of a cell surface receptor for ISG15 has yet to be discovered. Nevertheless, the actions of extracellular ISG15 have been most clearly tied to the induction of $\text{IFN}\gamma$ secretion by lymphocytes (31-35). Our findings in Chapter 6, demonstrated that susceptibility to acute LM infection in ISG15^{-/-} mice correlated with the significantly reduced expression of $\text{IFN}\gamma$ responses both in the spleen and in the blood. Although ISG15 deficient mice seemed to have limited impact of T cell responses to LM overtime, we did see that delivery of ISG15 as an adjuvant augmented LCMV-specific CD8 T cell protective immunity. We find this in accordance with previous data demonstrating that free soluble ISG15 can

induce lymphocytes to induce IFN γ responses (31-35). An explanation for this discrepancy could be that endogenous ISG15 is not essential for intrinsic T cell function, as there could be redundancy within the Type I IFN-induced responses. Therefore, by other unknown mechanisms the T cells are still able to get activated even if it is to a lesser extent. Alternatively, by using ISG15 deficient mice, our results provided evidence that ISG15 may facilitate or orchestrate the adaptive responses through enhanced dendritic cell maturation. Therefore, ISG15 may not only modulate T cells directly as a “cytokine-like” molecule, but may also foster cell-mediated adaptive immune response by augmenting APC functions. Further studies will be needed to elucidate the mechanism(s) underlying the adjuvant effects of ISG15. The evidence that ISG15 can be an effective adjuvant to drive potent CD8 T cell responses, support future studies to test its application in other infection models that require T cell immunity.

A major highlight of our study in Chapter 5 was demonstrating that ISG15 delivered as an immunoadjuvant generates responses independent of conjugation as an LRLRGG-mutant ISG15 also induced potent CD8 T cell responses. This suggested that the protection afforded by ISG15 was most likely not dependent on its conjugated form, but rather on free ISG15. However, we cannot exclude the functional properties of intracellular free ISG15 or conjugated ISG15 as it has been shown to also be biologically functional (32,37). We are currently pursuing their different association with modulating immunogenicity. Overall, the ability of free ISG15 (mutISG15) to induce superior antitumor responses (Chapter 5) highlights its potential to serve as an alternative potent ISG15 adjuvant. In addition, it emphasizes that developing new ways to increase the levels of free ISG15 may be a novel approach to treat cancer and other infectious diseases.

Free ISG15

To date, there are two mechanisms of action by which ISG15 contributes to the host response to infection: (i) conjugation dependent actions of ISG15 and (ii) the biological properties of ISG15 that are mediated by free ISG15. In this thesis, we demonstrate that the protection afforded by ISG15 can be independent on its conjugated form, free ISG15, supporting the notion that ISG15 perhaps functions as a cytokine. However, while this study provides the first *in vivo* evidence that free ISG15 delivered as an adjuvant contributes to host response during a tumor therapeutic or viral challenge model, it does not distinguish between the actions of extracellular and intracellular free ISG15. Therefore, further testing is needed to distinguish between the activity of intracellular and extracellular free ISG15. The identification of an ISG15 receptor(s) and *in vivo* blocking antibodies for ISG15 would be essential tools in defining whether the protection mediated by ISG15 in any infectious or tumor model is through the action of extracellular free ISG15. The availability of recombinant mouse ISG15 would also be a valuable tool to further establish a proof of concept. Overall, fully understanding the biological function of free ISG15 would allow us to fully harness its power to treat a variety of infectious diseases and cancers.

IL-33's and ISG15's Role on CD8 T Cell Memory Subsets

The establishment of immunological memory means that the immune system is able to respond with greater strength upon re-encounter with a same pathogen and is the basis for effective vaccination (38). The CD8 memory T cell population is heterogeneous, but perhaps the best characterized CD8 memory T cell subsets is the paradigm of central and effector memory cells (39-42). Central memory T (T_{cm}) cells localize to the lymphoid tissues and are capable of robust recall proliferation, whereas effector-memory T (T_{em}) cells, are predominately located in the peripheral sites and can quickly become cytolytic, but with limited proliferative capacity. There is still a controversy about which subset of memory CD8 T cells are most optimal for protective immunity. However, from a therapeutic point of view, the goal of successful vaccination might best be the induction of effector-like memory CD8 T cells to rapidly control infection and/or tumor growth. Recently, reports have begun to show that the effector-memory KLRG1⁺CD8⁺ T cell population can mediate potent protective immunity against certain pathogens (43-45) and might be optimal for immediate regression of established subcutaneous (s.c.) tumors (46). Our results showed that mice immunized with IL-33 and/or ISG15 induced robust expansion of Ag-specific effector memory CD8 T cells in the periphery, suggesting trafficking of activated CD8 T cells to the site of Ag stimulation. The high frequency of Ag-specific effector-memory cells in the periphery is consistent with the observation that effector-memory T cells can migrate to the site of infection and initiate immediate effector function (39). Our findings support the concept that vaccine-induced effector-memory CD8 T cell responses might be important memory CD8 T cell subsets for an effective therapeutic vaccine against tumors and other chronic infections (46). Furthermore, the quantity and quality of the effector memory T cells amplified by both our molecular adjuvants (IL-33 and ISG15) seemed to correlate with their efficacy in

the tumor or viral challenge models. Therefore, these potential correlates of immunity may suggest a different focus for vaccination strategies.

We are currently investigating the ability of IL-33 and ISG15 to generate T_{cm} immunity, since T_{cm} cells are important subsets that also mediate optimal protective immunity against pathogens and constitute the basis for vaccination (39-42,47,48). However, given that Murali-krishna *et al.* have reported that the initial magnitude (burst size or peak) of the CD8 effector T cell responses correlates to the long-term memory responses, suggest that IL-33 and ISG15 most likely can induce long-term immunity (49). Moreover, because preventative vaccines should induce long-lasting Ag-specific responses, we are further investigating the efficacy of these immunostimulatory adjuvants in the context of prophylactic vaccines and its establishment of long-lasting memory responses. Overall, understanding the mechanism of action by which either both IL-33 and/or ISG15 influences the expansion, or development of heterogeneous CD8 T cell populations in vaccines is an important area for further investigation.

IL-33's and ISG15's Role on the Humoral Responses

Antibodies are an important part of the host immune system to identify and neutralize infectious agents and have been the correlate of immunity for all currently successful licensed vaccines. Although CD8 T cells responses were important in our IL-33 study, we also examined the effects of IL-33 on B cell responses. The major significant difference between proIL-33 and mtrIL-33 was that proIL-33 was able to increase the HPV E7-antigen specific IgG binding levels. Full length IL-33's dual function property, to act not only as a cytokine, but also as a nuclear transcription factor, may explain the increase in antibody responses by proIL-33. Its nuclear localization may have effects on modulating the humoral immune responses. Thus, the specific transcriptional

targets of nuclear IL-33 are still unknown and must be further investigated. Another possibility for this outcome could have been due to the ability of proIL-33 to modulate the immune responses independent of its cognate ST2 receptor (11). We are currently pursuing understanding full-lengths association with modulating immunogenicity using ST2 knockout mice. Moreover, just recently published, Zhao *et al.* demonstrated that IL-33 promoted humoral responses to HBV during the pathogenic progress (48). They reported that IL-33 activated humoral immunity against HBV *in vivo* and *in vitro* by activating T follicular helper cells. This recent report confirms our findings that IL-33 can modulate humoral immune responses. Although the importance of our findings are not yet clear, these data suggests that IL-33 could also be useful in vaccine strategies aiming to achieve enhanced antibody responses and cellular immunity. In regards to ISG15, to the best of our knowledge, ISG15's role on the effect of antibody responses has never been examined and is an area of further investigation. In the future, in parallel with IL-33, we hope to study their immunoadjuvant properties on humoral responses using an influenza challenge model, a model which antibodies are known to be essential for protection.

IL-33's and ISG15's Effects Other Immune Cells (DCs, NK and CD4 T Cells)

It is well known that antigen presented by activated DCs and the type of production of polarizing cytokines they secrete can promote different fates on T cell development (50). First, we demonstrated that IL-33 could induce DC maturation by assessing the up-regulation of certain surface expression markers and their induction of proinflammatory cytokines, all of which may influence adaptive immunity. Mouse bone marrow derived dendritic cells (BMDCs) *in vitro* incubated with recombinant IL-33 up-regulated the DC maturation markers, CD80, CD86 and CD83 and secreted pro-

inflammatory T_H1 cytokines. The ability of IL-33 to induce phenotypic maturation of DCs *in vitro*, may explain IL-33's functional role on the impact of CD8 T cells observed in our study, recruiting the right type of immune cell infiltration necessary to facilitate both effective viral and tumor immunity. Regarding ISG15's effects on DC maturation, we observed that in ISG15^{-/-} mice, their DCs were impaired and unable to undergo DC maturation, suggesting a possible role for ISG15 in T cell activation and memory formation. Therefore, we are currently investigating both IL-33 and ISG15 roles on DC maturation and function during vaccination *in vivo*.

Considering that CD4 T cells play an important role in facilitating an effective adaptive immune response, by helping the activity of other immune cells (e.g. B cell antibody class switching, maximizing bactericidal and cytotoxic T cell activity) by releasing cytokines, we evaluated if our adjuvants can augment CD4 T_H1 cell responses. While ISG15 did not enhance Ag-specific CD4 T cells responses, IL-33, however did significantly increase the T_H1 CD4 T cell responses against HPV, HIV, and TB (Chapters 2,3,4). Although for years it was assumed that IL-33 mainly played a role in CD4 T_H2 immunity (given the early findings of the selective expression of ST2 by T_H2 but not Th1 cells) (6), our results intimated that mtrIL-33 *in vivo* might directly enhance T_H1 CD4 T cell responses. IL-33 as a DNA adjuvant can broadly expand T cell responses. An explanation for this could most likely be that only activated T_H1 CD4 T cells can express ST2 receptor, and in synergy with IL-12, local IL-33 will significantly augment a T_H1 response (22). This aspect requires further study. Taken together, our data shows that IL-33 can be both an effective CD4 and CD8 T cell vaccine adjuvant, while ISG15 seems to only enhance CD8 T cell-mediated responses. Nevertheless, it still needs to be completely determined whether ISG15 can modulate CD4 T cell responses, given that the models I used are mainly CD8 T cell-mediated. We plan to test ISG15 CD4 T effects

using other pathogen targets such as TB, where CD4 T_H1 responses are known to be critical for protective immunity. Although CD4 T cell help is not crucial for CD8 T cell priming, it is believed to be essential in the expansion of secondary responses of memory CD8 T cells (39,51,52). Thus, we are currently determining if IL-33 or ISG15 can induce effective CD8 T cell responses independent of CD4 responses during the course of homologous prime-boost vaccination.

Finally, it is likely that other immune cells may have accounted for the observed enhancement in T_H1 immunity. For instance, both IL-33 and ISG15 have been shown to activate Natural Killer (NK) cells to secrete IFN γ (8,23). Nevertheless, further studies will be needed to elucidate under what conditions both IL-33 and ISG15 promotes T_H1 and CD8 T cell immunity; to determine their regulatory networks bridging the innate with the adaptive immune responses.

IL-33's and ISG15's Application for Other Disease Targets and Cancer

Given these significant findings, we went on to observe in a preclinical setting, that IL-33 would also increase the Ag-specific CD4 and CD8 T cell responses when co-delivered with a DNA vaccine encoding either a HIV, flu, malaria or TB target antigens (Chapters 2,3,4 and unpublished data). We are currently examining the efficacy of IL-33 in a malaria, influenza, and TB challenge models. On the other hand, we reported that ISG15^{-/-} mice are highly susceptible to infection with the model pathogen, *Listeria monocytogenes* (LM), and display significantly reduced production of IFN γ . Therefore, with the recent finding observed by Bogunovic *et al*, that individuals with mutations in *Isg15* are more susceptible to infection by the intracellular bacterium *Mycobacterium tuberculosis* (Mtb) (36), we are next evaluating ISG15's application in a TB-infection

model. Overall, these findings significantly highlight the important role for both IL-33 and ISG15 as effective adjuvants to be incorporated into future vaccines targeting an array of microbial infections.

The important roles IL-33 and ISG15 may play in cancer is only beginning to surface. For instance, three recent studies have highlighted the important role of IL-33 in human cancer and tumor mouse models (Lung, melanoma, cervical, breast and carcinoma) and have shown that IL-33 can drive antitumor CD8 T cell responses (27,53-58). For ISG15, its role in protective tumor immunity has only begun to be appreciated (57). In accordance with our data, Burks *et al* just recently demonstrated that free ISG15 could induce an antitumor immune response *in vivo* and *in vitro* against breast cancer (58). They reported that extracellular free ISG15 suppresses breast tumor growth and increased NK cell infiltration into xenografted breast tumors in nude mice. Clearly, my studies provided stronger evidence for IL-33's and ISG15's beneficial role in tumor immunity and their utility to be used as cancer immunotherapies. My studies showed that a HPV DNA vaccine plus our adjuvant combinations (IL-33 and/or ISG15) induced great control or complete tumor regression in a therapeutic HPV-associated murine tumor challenge model. More studies using ISG15^{-/-}, UBE1L^{-/-}, ST2^{-/-} and IL-33^{-/-} mice will help further elucidate the significant roles ISG15 and IL-33 play in the host antitumor responses. Given the recent studies (as mentioned above) showing that IL-33 and ISG15 as immunoadjuvants can augment Ag-specific T_H1 and CD8 T cell immune responses, its role in enhancing tumor surveillance and antitumor immunity is worth continued investigation. In the future, we plan to study both IL-33's and ISG15's specific role in the tumor microenvironment and surveillance process.

Similarities and Differences Between IL-33 and ISG15 Adjuvant Properties

Both IL-33 and ISG15 delivered as vaccine adjuvants enhanced potent Ag-specific polyfunctional CD8 T cell tumor and viral immunity. Both adjuvants induced high frequencies of effector CD8 T cells responses compared to the non-treated adjuvant DNA vaccine groups. In contrast to IL-33, ISG15 did not significantly enhance the CD4 T cell responses. Therefore, IL-33 is capable of augmenting broader T cell responses. However, given ISG15's ability to modulate the adaptive responses (increasing vaccine-induced IFN γ responses), suggests that ISG15 can potentially function like a cytokine similar to IL-33. In addition, besides their effects on CD8 T cells, our results may propose that the adjuvants effects of IL-33 and ISG15 may also be through DC activation and maturation. Further studies are warranted to determine how IL-33 and ISG15 orchestrate the adaptive responses through enhanced DC maturation. Overall, I have provided evidence of their potential relevance for the design of future T-cell based vaccines against cancers and infections requiring such desired responses. Since both IL-33 and ISG15 adjuvants can affect the immune responses in different ways, such as eliciting a broad range of immunological responses (humoral vs. cellular immunity or both), it is important to choose a candidate adjuvant for future specific vaccine formulations. The results of the similarities and differences between IL-33 and ISG15 are summarized in **Table 7.1**.

Combination of Adjuvants

Adjuvants are critical components of many vaccines and will continue to be essential components for the development of future vaccines for the treatment of infectious diseases and cancers. However, deciding how to select to the best vaccine adjuvant for enhancing protective immunity will depend on the disease in question. Consequently, no single adjuvant is capable of stimulating broad and robust protective immune responses required to fight chronic infectious diseases and cancers. Therefore, it is believe that a future design of vaccines will focus on incorporating multiple adjuvants as a way to enhance multiple immunological parameters and improve vaccine efficacy. Recent studies have shown that using multiple adjuvants in combination can synergistically enhance or improve the immune responses to vaccines. For example, the combination of toll-like receptor agonists can enhance DC function, the induction of CD8 T cell responses, and antibody responses (59,60). The literature has even shown evidence of combination adjuvants containing cytokines like IL-12, IL-2 and GM-CSF increasing specific CTL and IFN γ -secreting T cell responses (4,59-61). Therefore, with this knowledge, in the future, we hope to coadminister IL-33 and ISG15 in combination or simultaneously in a vaccine setting. We also plan to compare their efficacy individually or in combination to other T_H1 inducing cytokines, such as IL-12 and IL-18. In addition, we plan to test both IL-33 and ISG15 in combination with other licensed adjuvants (alum, ASO4, or MF59) as a goal to enhance effective antibody and cellular responses required to induce protective immunity in each particular disease.

Although the combination of adjuvants may be the way forward in vaccine design to elicit robust and broad protective immune responses, such an approach might not be enough to treat cancer or chronically infected patients, where overcoming the well-

established immunosuppressive environment can be challenging. Therefore, an alternative, will be to administer vaccines in combination with other therapeutic interventions (e.g. immunotherapy), such as monoclonal antibody immune checkpoint inhibitors (e.g. PD1, PDL1, LAG-3, and/or TIM-3). Identifying the appropriate combination of adjuvants (double combination therapy) plus therapeutic treatments (triple combination therapy) may offer tremendous opportunities for improving the efficacy of future vaccines to treat against complex chronic diseases and cancer.

Clinical Application

It may be an investing effort into harnessing the power of IL-33 and ISG15 in the clinical setting. For instance, a recent study examining the expression of IL-33 in cervical tissue of patients with different stages of HPV cervical disease showed that lower levels of IL-33 in cervical tissue were associated with more severe stages of HPV-induced dysplastic change (55). Furthermore, an important study by Casanova and colleagues, have shown that humans lacking ISG15 develop mycobacterial diseases (35). Therefore, it is conceivable to suggest that delivery of IL-33 or ISG15, like other cytokines IL-2 or IFN γ , could be used to help fight viral and bacterial infections, as well as cancer in the general population. However, this hypothesis remains to be tested. In order to move these adjuvants into clinical studies, their immunogenicity and efficacy must be tested in other animal models. In the future, we to hope to immunize non-human primates with our adjuvants along with selected target antigens (e.g. HPV or HIV) study to determine immunogenicity.

Closing Remarks

In this thesis, I exclusively show for the first time that IL-33 and ISG15 can be promising molecular adjuvants at improving CD8 T cell immunity in a vaccine setting. The exciting evidence that IL-33 and ISG15 can induce protective immunity in both infectious and cancer models, supports their development as immunoadjuvant candidates for enhancing the potency of both prophylactic and therapeutic DNA vaccines (**Figure 7.1**). This knowledge opens new avenues for harnessing the power of IL-33 and ISG15 as immunostimulatory adjuvants for future novel vaccines against diseases that require T_H1/CD8 T cell protective immunity such as TB, malaria, HIV, and even cancers. In the end, although the immune system may never win the war against disease, but with effective adjuvants like IL-33 and ISG15, it might just avoid some mortal battles.

FIGURES

Table 7.1. Similarities and Differences between IL-33 and ISG15 vaccine-induced adjuvant properties

Adjuvant properties	IL-33	ISG15
Induced Ag-specific T _H 1 responses (IFN γ)	Yes	Yes
Induced Ag-specific T _H 2 responses (IL-4)	No	n.d.
Increased polyfunctional Ag-specific CD4 T cells (secreting IFN γ , TNF α and IL-2)	Yes	No*
Increased polyfunctional CD8 T cell (secreting IFN γ , TNF α and IL-2)	Yes	Yes
Enhanced Ag-specific CD8 T cells with a cytolytic phenotype	Yes	Yes
Increased T bet expression in CD8 T cells	Yes	n.d.
Increased Ag-specific effector-memory-like CD8 T cell responses	Yes	Yes
Induced long-term central memory T cell responses	n.d.	n.d.
Increased NK cells	n.d.	No
Play a role in DC activation and maturation	Yes (<i>in vitro</i>)	Yes (<i>in vivo</i>)**
Increased Ag-specific humoral responses (IgG)	Yes	n.d.
Antitumor Protective Immunity	Yes	Yes
Antiviral Protective Immunity	Yes	Yes
Increased antibacterial Ag-specific CD4 and CD8 T cell immune responses	Yes	n.d.
Cytokine/DAMP-associated molecule	Yes	maybe
Effective T cell vaccine adjuvants	Yes	Yes

*Although in our vaccine models ISG15 did not enhance CD4 T cell responses, further testing is needed to establish its effects on CD4 T cells, if any.

**Although the vaccine-induced DC were not assessed, ISG15-/- highlights a critical role for DC activation and maturation

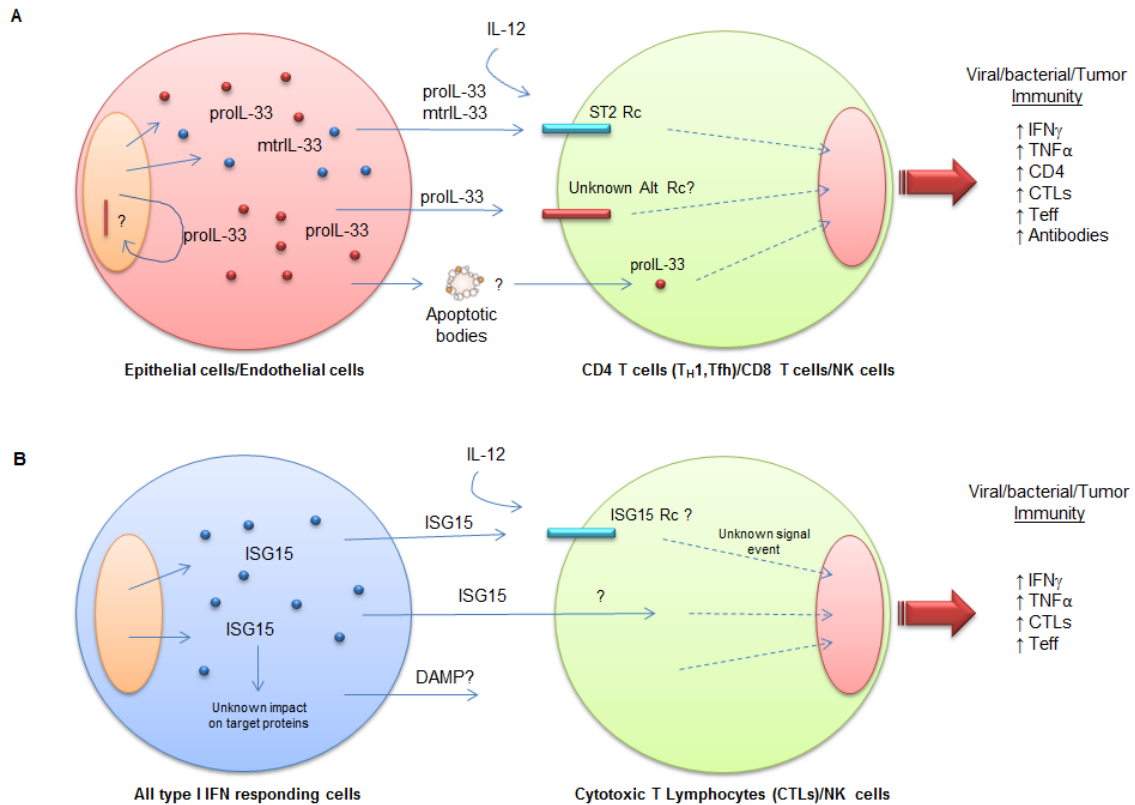


Figure 7.1. The potential biological mechanisms of the adjuvant properties of IL-33 and ISG15 on immune cells to induce viral, bacterial, and tumor immunity. These observed effects are determined by the specific cells targeted and the associated cytokine network. (a) IL-33 as a cytokine has dual-function: acting as a cytokine and localizing in the nucleus which functions are unclear. The administration of both full-length IL-33 (proIL-33) and mature IL-33 (mtrIL-33) can have cellular activities on NK/NKT, CD4 and effector CD8 T cells which produce Th1-associated protective immunity. Also potentially accompanying, APCs produce and secrete IL-12, which then induces expression of ST2 on NK/NKT cells and CD8⁺ T cells, permitting IL-33 to induce Th1-associated cytokine production. It is unclear whether Th1 CD4⁺ T cells are able to upregulate ST2, however, IFN γ production by other activated immune cells likely leads to their amplification which then can help activate antiviral and tumoricidal immunity. Interestingly, recent evidence hints at a new activity for proIL-33 to activate cells independent of the ST2 receptor. Does proIL-33 bind to another unknown receptor? Or is proIL-33 taken up by cells by apoptotic bodies caused from necrotic cells, which in turn, allows proIL-33 to migrate into the nucleus of the new cell and facilitating an immune response? These mechanisms are unknown. Moreover, data has demonstrated that proIL-33 can elicit antigen-specific antibodies, yet its role in protection against infectious pathogens remains to be determined. (b) Type I IFNs stimulates the production of ISG15 usually after infection or by tissue damage. Normally, ISG15 conjugates to target proteins by a method known as ISGylation to control viral infection. However, ISG15 can also exist into other forms: free intracellular ISG15 and free extracellular ISG15 which are depicted here. Little is still known about the properties of conjugated ISG15 form, but even less is known about the free forms of ISG15. Interestingly, recent evidence (along with what is presented in this thesis) highlights that free extracellular ISG15 may potentially act as a cytokine and induce Th1 immune responses. Free soluble ISG15 has now been described as having cellular activities (adjuvant properties) on NK cells and CD8 T cells. ISG15's immunological properties suggest it might have a receptor, however, no ISG15 receptor has been discovered. It is believed that in synergy with IL-12, ISG15 can further augment Th1 immunity. It is unclear if ISG15 works as a DAMP-associated molecule (increasing DC function or maturation) or if it is engulfed by the cells through unknown process to modulate adaptive immune responses. It is known that free intracellular ISG15 has immunomodulatory properties, but the molecular mechanisms behind it are still unknown. *Notes:* DAMP, damaged-associated molecular pattern molecule; NK, natural killer cells; INF, interferon; Alt, alternative; Rc, receptor; Teff, effector memory CD8 T cells; Tfh, CD4 T follicular helper cells

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